Exhibit 3

UNITED STATES DISTRICT COURT
SOUTHERN DISTRICT OF NEW YORK

IN RE: Acetaminophen – ASD-ADHD Products Liability Litigation **This Document Relates To: All Actions**	**22:md-3043 (DLC)**

EXPERT REPORT OF ROBERT M. CABRERA, Ph.D.

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The purpose of this report is to examine the developmental and reproductive toxicity of acetaminophen, also known as paracetamol, and sold as a medication used to treat fever and pain under common brand names such as Tylenol and Panadol. The capacity of this substance and its metabolites to cause general toxicity, neurotoxicity, and development and reproductive toxicity will be addressed using standard methodology for causality analysis.

The opinions set forth herein are expressed with a reasonable degree of scientific certainty. Moreover, the opinions stated in this report are consistent with the totality of available evidence on acetaminophen and its metabolites. This evidence includes both public and confidentially disclosed data, reports, and presentations.

QUALIFICATIONS

Robert M. Cabrera, Ph.D.

I have studied and conducted birth defect research for over 20 years, as indicated in my curriculum vitae, attached hereto as Appendix A. I work in the field of science called teratology. Teratology is the scientific study of abnormalities, malformations, and developmental disorders that occur during prenatal development. It is a branch of developmental biology and embryology that focuses on understanding the causes, mechanisms, and outcomes of birth defects. The field of teratology examines the impact of various factors on the development of embryos and fetuses, including genetic abnormalities, environmental agents, maternal health conditions, and drug exposures. By studying these factors and their effects, teratologists aim to identify the causes of birth defects and develop strategies for prevention and treatment.

Teratology encompasses overt toxicity, including environmental or chemical exposures that produce death of an embryo or fetus, and a wide range of abnormalities, such as structural defects (e.g., cleft lip or heart malformations), growth abnormalities (e.g., small for gestational age or intrauterine growth restriction), and functional impairments (e.g., neurodevelopmental disorders or sensory disorders). There are termed the four manifestations of deviant development (i.e., death, malformations, growth retardation, and functional deficits). Researchers in this field often use animal models, such as mice, rats, rabbits, or zebrafish, to investigate the underlying mechanisms of abnormal development and to test potential interventions. In the section below titled “Embryology and Teratology” I provide additional details on these subjects.

The goal of teratology is to enhance our understanding of normal and abnormal development, promote healthy pregnancies, and minimize the risk of birth defects through education, research, and public health initiatives. My primary research interests include the teratogenicity of environmental exposures and pharmaceuticals, and the study of human and animal teratogens has been central to my training, academic research, and teaching activities.

I am currently an Associate Professor in the Center for Precision Environmental Health, Department of Cellular and Molecular Biology, at Baylor College of Medicine in Houston. I maintain an adjunct appointment with San Jacinto College, Department of Biology, in Houston. I established the online Nutrition course at The University of Texas at Austin, where I also held an adjunct appointment with University Extension. My prior appointments in Austin included Instructor at Concordia University, Manager for Stem Cell Research at the Dell Pediatric Research Institute, and a Faculty Lecturer and Research Scientist in the Department of Nutritional Sciences at The University of Texas at Austin. I have

a Ph.D. in Medical Sciences from Texas A&M University Health Science Center, where I studied genetic, immunologic, and pharmaceutically induced congenital malformations in experimental animal models and humans.

I obtained my undergraduate degree at Texas A&M University in College Station (B.A., 1995). I have worked in private industry laboratories doing genetic sequencing of humans, animals, plants, bacteria, and viruses, and I have developed commercial processes for using high-content screening of genetic polymorphisms and gene expression analyses for clinical applications. I enrolled in graduate studies at Texas A&M University Health Science Center and completed my graduate training at the Institute of Biosciences and Technology in Houston, Texas (Ph.D., 2006). My graduate mentor was Professor Richard H. Finnell, who is a leader in the field of teratology and has worked in the area of birth defects research for over 35 years as a board-certified medical geneticist and as an established animal research investigator.

A major theme of my work is the complex and often reciprocal interrelationships between maternal immunity and birth defect risks. My graduate school research focused on developing and utilizing mouse models for birth defect research and developing novel laboratory tests for measuring risk factors for birth defects from the California Birth Defects Monitoring Program.[1] This included animal mechanistic studies using genetic manipulation, toxicant exposures, and studies on valproic acid (Depakote), a recognized human teratogen, and one of the most widely prescribed anti-epileptic medications. My post-doctoral work included the study of associations between mothers with folate receptor autoimmunity and the risk of neural tube defects (NTDs). These studies examined paradigms in developmental biology, including the reduced risk for neural tube defects through supplementation with folic acid, and disrupted fertility, placental function, and embryo-fetal development due to autoimmunity. In several studies, I found that maternal IgG or IgM autoantibodies to folate receptors increase the risk for NTDs. I also determined IgM autoantibodies were associated with risks for cleft palate and NTDs in a Norwegian population. Specifically, my postdoctoral research involved the continued study of birth defects in expectant mothers from the Norwegian Mother and Child Cohort Study[2] (MoBa) and the Danish National Birth Cohort Study.[3] The Norwegian Mother and Child Cohort Study (MoBa) is a large population-based study conducted in Norway. It is one of the largest pregnancy cohort studies in the world and has been ongoing since 1999. The primary aim of MoBa is to investigate various factors that influence health and development in pregnancy, infancy, childhood, and beyond. The study involves the participation of pregnant women in Norway who are recruited during their routine ultrasound examination at approximately 17-18 weeks of gestation. The women provide information through comprehensive questionnaires during pregnancy and after birth. The Danish National Birth Cohort (DNBC) Study, also known as "Danish Mother and Child Cohort Study," is a large-scale population-based study conducted in Denmark. The DNBC recruited pregnant women from 1996 to 2002, enrolling over 100,000 participants. The study involved collecting detailed information through comprehensive questionnaires completed by the pregnant women at different stages of pregnancy, during birth, and following birth. Additionally, biological samples such as blood and urine were collected from the participants. I analyzed autoimmune

[1] Cabrera et al. Autoantibodies to folate receptor during pregnancy and neural tube defect risk. J Reprod Immunol. 2008 Oct;79(1):85-92. doi: 10.1016/j.jri.2008.08.002. Epub 2008 Sep 18. PMID: 18804286; PMCID: PMC3998370.

[2] Boyles et al. Association between inhibited binding of folic acid to folate receptor alpha in maternal serum and folate-related birth defects in Norway. Hum Reprod. 2011 Aug;26(8):2232-8. doi: 10.1093/humrep/der144. Epub 2011 May 15. PMID: 21576080; PMCID: PMC3137385.

[3] Bille et al. Autoantibodies to folate receptor alpha during early pregnancy and risk of oral clefts in Denmark. Pediatr Res. 2010 Mar;67(3):274-9. doi: 10.1203/PDR.0b013e3181cbd564. PMID: 19952865; PMCID: PMC2909840.

factors in patients from these populations as a risk factor for various birth defects, including neural tube defects and cleft lip and palate. These study populations have also been used to investigate other risk factors for birth defects and functional deficits, including autism spectrum disorder (ASD) and attention-deficit hyperactivity disorder (ADHD).

Upon completion of my postdoctoral research, I worked for a non-profit organization, the Texas Institute for Genomic Medicine (TIGM). TIGM was a public-private partnership that created and distributed mouse embryonic stem cell clones with genes inactivated (knockouts) to investigators around the world. As a Research Scientist at TIGM, I created animal models of human diseases and developed embryonic stem cell models for testing developmental and biological select agents and toxins. My project proposal was awarded $12.25 million dollars through the Defense Threat Reduction Agency (DTRA, US DOD) to study select agents using the testing models I developed. TIGM was subsequently assimilated entirely into the Texas A&M University System. I transitioned to The University of Texas at Austin and the Dell Pediatric Research Institute as a Research Fellow, where I continued my research on mouse and embryonic stem cell models for reproductive and developmental toxicity testing. During my academic career, I have written chapters on the use of functional genetics in developmental toxicity testing and have published over 30 peer-reviewed articles on teratogens and birth defects. I am currently developing and maintaining National Institutes of Health and National Institutes of Mental Health funded research programs that lead the field in developmental toxicity testing of anti-retroviral therapies (ART) at Baylor College of Medicine. In this capacity, I have provided oral and written reports to national (FDA, CDC) and international (WHO) regulatory agencies on the prevention of birth defects associated with ART. I am also on the editorial advisory board for Birth Defects Research, the peer-reviewed journal of the Birth Defects Research and Prevention Society (Teratology Society). Through my experience in teratology research, I have examined and processed hundreds of litters, and thousands of fetuses, for external, visceral, and skeletal malformations. I actively conduct and participate in human epidemiology and animal teratology studies that focus on determining the mechanisms of birth defects. This includes the use of clinical chemistry, analytical chemistry, immunological testing and immunodiagnostics, pathology, and hematology. I also conduct and participate in environmental chemical and pharmaceutical studies for teratogenicity testing using cellular, tissue, organ, and *in vivo* models, including zebrafish, genetically altered mice, and inbred mice.

My teaching activities include lectures, laboratory training and instruction, directing projects, and mentoring or assisting student researchers. My current teaching load includes professional student and graduate student lecture/seminar courses in the field of medical biochemistry, in the School of Health Professionals, and guest lecturing in developmental biology and nutritional sciences. I am also a course instructor at Baylor College of Medicine and participate in the development/design of the medical biochemistry curriculum.

Based on my qualifications, training, and experience, I am competent to set forth the opinions below.

SUMMARY OF OPINIONS

After performing a Weight of Evidence (WoE) analysis: reviewing the chemical profile of acetaminophen (APAP), regulatory adverse outcome pathway (AOP), and the preclinical, *in vitro, ex vivo*, and *in vivo* studies for potential reproductive, developmental, and neurodevelopmental effects, in the context of "therapeutic" doses of APAP use;

I hold the following opinions to a reasonable degree of scientific certainty:

I. Acetaminophen produces oxidative damage, reproductive, developmental, and neurodevelopmental toxicity at clinically relevant dosages, including neurodevelopmental disorders, such as autism spectrum disorder (ASD) and attention deficit hyperactivity disorder (ADHD).

II. Therapeutic dosages of acetaminophen taken by pregnant woman are sufficient to cause neurotoxicity, neurodevelopmental disorder, ASD, and ADHD in exposed offspring.

III. There is a historic and growing body of research and evidence in the scientific and medical community that support the reproductive, developmental, and neurodevelopmental toxicity of acetaminophen exposures.

Additional opinions and subsidiary opinions are also expressed throughout this report.

MATERIALS CONSIDERED

In order to convey my opinions, I will present the relevant data and available information:

1. My knowledge, training, and experience as set forth above and in my annexed curriculum vitae;

2. Disclosures provided by Defendant(s) including responses to discovery demands identifying research, presentations, and reports on acetaminophen;

3. The scientific and medical literature cited throughout this report;

4. Governmental and regulatory publications cited throughout this report;

5. Other expert reports submitted by Plaintiffs, including expert reports prepared by Drs. Andrea Baccarelli, and Brandon Pearson; and

6. Depositions and documentary disclosure provided in these cases.

SUMMARY OF EVIDENCE

The following summary is based on a systematic review of relevant studies on acetaminophen toxicity and guidance documents on reproductive, developmental, and neurodevelopmental toxicity testing. **The primary question to be answered: does acetaminophen exposure during pregnancy cause functional deficits in offspring, specifically, ASD and ADHD?** The population studied is pregnant females. The exposure of interest is pharmaceutical intake of acetaminophen during gestation. These exposures are compared to females unexposed or as a dosed-response exposure, and the outcomes examined include manifestations of deviant development, generally, and functional deficits, specifically, ASD and ADHD. The protocol followed is based on Weight of the Evidence (WoE) for examining study quality and Bradford Hill, applied for significant associations, to evaluate causality. A comprehensive literature search was conducted, and relevant information and studies are summarized below and detailed in the following report. Background information is provided and reviews the pharmacokinetic, mechanism of action, and associated toxicities with acetaminophen exposures. Both primary reports from animal model testing and epidemiology studies were reviewed. In addition, meta-analyses were reviewed, and alternative explanations were considered. I have interpreted the collective totality of evidence, identified associations, strengths, and limitations in studies, and draw the following conclusion: **based on the totality of evidence, acetaminophen exposure during pregnancy causes ASD and ADHD in offspring.**

Summary of Toxicological Endpoints and Risk Characterization for Acetaminophen

Topic	Key Information, Data, and Evidence
Pharmacokinetics of Acetaminophen (APAP)	•Available in tablet, capsule, intravenous infusion, and liquid formulations (PDR) ·Bioavailability ranges by formulation from 85-98% ·Immediate release peak plasma concentrations occur within 30-60 minutes with oral administration and range from 7.7 to 17.6 mcg/mL (~50-116 µM) after a single 1,000 mg dose and 7.9 to 27 mcg/mL (~52-178 µM) at steady state after 1,000 mg every 6 hours in adult patients. ·In children 2 to 7 years of age, acetaminophen (12 mg/kg) achieved maximum concentration (14.6 +/- 2.6 mcg/mL) (~96 µM) within 0.55 +/- 0.08 hours.
Therapeutic Mechanism of Action	• **APAP is the only aniline analgesic still in use, all other members of this drug class are banned.** · Human therapeutic dose = 1g (16.7mg/Kg in 60Kg human), mouse median effective dose as antinociceptive (ED_{50}) = 125.4mg/Kg, rat ED_{50} = 154.8mg/Kg (Guash 1990). ·Several therapeutic mechanisms have been reported, including cyclo-oxygenase (COX) inhibition, serotonergic (5-HT) signaling, and metabolite mediated (AM404) cannabinoid signaling. ·AM404 activates TRPV1, a ligand at cannabinoid receptors (CB1) and an inhibitor of endogenous cannabinoid (anandamide) uptake.
General Toxicity	• **One of the most common causes of poisoning worldwide. (PubChem)** • **Most common cause of acute liver failure in the United States. (PDR)** ·Risk of toxicity increases with serum ≥150 µg/mL at 4 hours post ingestions, termed the "150" treatment line (~990 µM). • **Increased risk of acute oral overdose at intake ≥ 150 mg/kg (~7.5 g in adults)** ·Limited margin of safety comparing daily therapeutic 4g (1g/6 hours) and risk of acute toxicity at ≥ 7.5g. • **The toxic effects of APAP are due to a metabolite (NAPQI). (PubChem)** ·Cytochromes (P450) CYP2E1 and CYP3A4 convert ~5% of APAP to NAPQI. ·NAPQI reacts with sulfhydryl groups on proteins and with glutathione (GSH). ·GSH is an essential antioxidant; decreased GSH can result in protein, cell, tissue, and organ damage. • **Lethal Dose (LD) references for reviewed animals** ·Mouse LD50 = PO 945-1212 mg/kg (Guash 1990), IP 800mg/Kg (Mancini 1980) ·Rat LD50 = PO >4000 mg/kg (Guash 1990), IP 1580mg/Kg (Mancini 1980)

Topic	Key Information, Data, and Evidence
Genotoxicity and Carcinogenicity	• **Animal models and human studies report APAP increases leukemia risk (NTP, Weiss et al. 2005)** • **APAP is associated with an estimated two-fold increased risk (point estimate range: 1.5-2.3) of myeloid leukemias in humans. (Weiss et al. 2005, Walter et al. 2011)** • **There was equivocal evidence of carcinogenic activity of acetaminophen in female rats (F344/N) based on increased incidences of mononuclear cell leukemia. (NTP)** ·Equivocal evidence of carcinogenic activity describes studies that are interpreted as showing a marginal increase of neoplasms that may be chemically related. (NTP)
Adverse Outcome Pathway (AOP) for Neurodevelopmental Impairment	• **APAP readily crosses the placenta and the fetal blood-brain barrier.** ·Therapeutic doses of APAP produce the toxic metabolite NAPQI in sufficient concentrations to produce oxidative stress adverse effects in every region of the brain. • **Oxidative stress from NAPQI reduces/depletes the antioxidant glutathione (GSH)** ·Oxidative damage to developing neural tissues (dose and time dependent), results in dysregulated proliferation, differentiation, and patterning of neurons and neural cells. • **Therapeutic doses of APAP produce the metabolite AM404** ·Increased anandamide/endocannabinoid signaling. ·Disruption of endocannabinoid signaling during brain development results in neurodevelopmental impacts, including cognitive impairment of learning and memory, behavioral problems, and attention difficulties. • **Animal studies demonstrate that perinatal exposure to APAP at therapeutic doses cause significant changes in brain biochemistry, learning, and behavioral testing.** ·**Humans with ASD and ADHD have biomarkers, brain tissue patterns, and reproductive impacts consistent with outcomes observed in *in utero* exposure to APAP in animal models.**
Reproductive, Developmental, and Neurodevelopmental Toxicity	• **Epidemiological studies and meta-analysis report that use of APAP during pregnancy significantly increases risk of ASD and ADHD in offspring.** ·Studies measuring APAP in cord blood or meconium replicate and support dose-effects on neurodevelopment and reject recall bias. ·Confounding by indication or unknown confounding are proposed as alternative explanations for reported associations/risks. ·Studies comparing APAP and other analgesics to ASD or ADHD do not support confounding by indication. • **Exposing mice or rats to human equivalent doses of APAP during neural development causes altered behavior consistent with ASD and ADHD.** ·Reproductive, developmental, and neurodevelopment impacts on animals occur in the absence of indication. • **15 studies in animal models investigated the effects of early-life exposure to acetaminophen on behavior, with 14 showing impaired learning and altered behaviors.** • The one study that found "no evidence" showed low doses of acetaminophen improved learning. This study was conducted at sub-therapeutic dosing.

Weight of Evidence, Bradford Hill, and Causal Interactions	• The Weight of Evidence (WoE) analysis on available human and animal studies demonstrates "clear evidence" of neurodevelopmental toxicity at "therapeutic" doses. APAP and the metabolite NAPQI increase (1) oxidative damage, (2) DNA oxidation, (3) protein adduct formation, and thereby cause (4) epigenetic and associated gene expression changes, (5) mitochondrial and cellular toxicity, and (6) reproductive, developmental, and neurodevelopmental toxicity. • Based on Bradford Hill, there is sufficient data to support causal interactions between APAP and reproductive toxicity and neurodevelopmental toxicity, including ASD and ADHD. • Strength of Association: The meta-analyses indicate statistically significant associations between acetaminophen exposure and ASD and ADHD risk. These reports meet the criterion of strength of association. • Consistency: The meta-analyses combine data from multiple cohort studies, indicating a consistent pattern of association between acetaminophen exposure and ASD and ADHD risk across different populations. The consistency criterion is met. • Specificity: APAP exposure is common and is associated with other toxicities, such as being the number one cause of acute liver failure in the USA. ASD and ADHD can be caused by other environmental or chemical exposures, such as *in utero* valproic acid exposure. The specificity criterion is not fully met. • Temporality: The exposure to acetaminophen (during pregnancy) precedes the outcome (development of ASD or ADHD) temporally, supporting a temporal relationship. The temporality criterion is met. • Biological Gradient: The meta-regression analyses indicate that the association between APAP exposure and ASD risk increased with the child's age at follow-up and the mean duration of exposure. This supports a biological gradient, as a longer duration of exposure and older age were associated with a higher risk of ADHD. The biological gradient criterion is met for ASD. Meta-analyses did not explicitly report a dose-response relationship between APAP exposure levels and the risk of ADHD. Two studies looked at APAP in meconium, one supporting a dose-response interaction between APAP and ADHD. Another study looked at cord blood and supported a dose-response interaction. The biological gradient criterion for ADHD is supported. • Plausibility: There is biological plausibility for the association, as APAP can cross the placental barrier and affect fetal neurodevelopment. The published adverse outcome pathway (OECD 20) also supports a causal interaction between APAP and oxidative stress and neurodevelopmental adverse outcomes. The neurodevelopmental effects of acetaminophen on ADHD risk are biologically plausible and supported.

Topic	Key Information, Data, and Evidence
	• Coherence: The association between APAP exposure and ASD and ADHD is coherent with the existing knowledge of oxidative stress and potential for hepatotoxicity and neurotoxicity with APAP exposures. The coherence criterion is met. • Experiment: Conducting experimental studies on women of childbearing age to test chemical causality of birth defects or functional deficits is not ethically feasible, as it would involve exposing pregnant women to potentially harmful substances. Animal models report dose-responsive reproductive toxicity, dose-responsive developmental toxicity, and neurodevelopmental toxicities with perinatal APAP exposures. This criterion is met with animal models. • Analogy: There are analogies with other substances that are known to have neurodevelopmentally toxic effects during pregnancy, including mercury and valproic acid, supporting a common oxidative stress mechanism for mercury, like APAP, and chemical-pharmaceutical causes of ADHD and ASD.

Key Terms: APAP: paracetamol/acetaminophen (N-acetyl-p-aminophenol); NAPQI: APAP metabolite N-acetyl-p-benzoquinone imine; AM404: APAP metabolite N-arachidonoylphenolamine; COX: cyclooxygenase; 5-HT: serotonin; DART: developmental & reproductive toxicity; LD50: median lethal dose (dose that results in death of 50% of animals); OECD: The Organization for Economic Co-operation and Development; WoE: weight of evidence; NTP: National Toxicology Program; PDR: The Physicians' Desk Reference (acetaminophen - Drug Summary), PDR.net (online); ASD: Autism Spectrum Disorder; ADHD: Attention Deficit Hyperactivity Disorder.

- Guasch et al. Pharmaco-toxicological effects of acetaminophen in rodents. Battery of tests to screen potential analgesic acetaminophen derivatives. Methods Find Exp Clin Pharmacol. 1990 Mar;12(2):141-8. PMID: 2319838.

- Mancini, R et al. Developmental susceptibility to acetaminophen toxicity. Res Commun Chem Pathol Pharmacol. 1980 Mar;27(3):603-6. PMID: 7384649.

- Weiss et al. Opposing effects of aspirin and acetaminophen use on risk of adult acute leukemia. Leuk Res. 2006 Feb;30(2):164-9. doi: 10.1016/j.leukres.2005.06.023. Epub 2005 Aug 11. PMID: 16099041.

- Walter et al. Long-term use of acetaminophen, aspirin, and other nonsteroidal anti-inflammatory drugs and risk of hematologic malignancies: results from the prospective Vitamins and Lifestyle (VITAL) study. J Clin Oncol. 2011 Jun 10;29(17):2424-31. doi: 10.1200/JCO.2011.34.6346. Epub 2011 May 9. PMID: 21555699; PMCID: PMC3107756.

- National Toxicology Program. NTP Toxicology and Carcinogenesis Studies of Acetaminophen (CAS No. 103-90-2) in F344 Rats and B6C3F1 Mice (Feed Studies). Natl Toxicol Program Tech Rep Ser. 1993 Jan;394:1-274. PMID: 12637965.

METHODOLOGIES EMPLOYED

In performing my analyses, I have brought to bear the accepted methodologies that are commonly employed in medical and scientific disciplines, to assess general causation. These include the general principles of a) genetics and clinical genetics, b) toxicology, c) pharmacology d) epidemiology, e) epigenetics, and f) teratology and developmental toxicology.

Causality is a concept that has been studied for centuries. One of the most influential theories on causality was developed by Sir Bradford Hill in 1965. Hill's criteria for establishing causality have become known as the Bradford Hill criteria in scientific, medical, and legal writings. These criteria provide a framework for determining whether or not there is a causal relationship between two variables. The Bradford-Hill Criteria are as follows:

1. **Strength of association**: A small association does not mean that there is not a causal effect, though the larger the association, the more likely that it is causal.
2. **Consistency:** Consistent findings observed by different persons in different places with different samples and techniques strengthen the likelihood of an effect.
3. **Specificity**: Causation is likely if there is a very specific population at a specific site and disease with no other likely explanation. The more specific an association between a factor and an effect is, the bigger the probability of a causal relationship.
4. **Temporality**: The effect has to occur after the cause (and if there is an expected delay between the cause and expected effect, then the effect must occur after that delay).
5. **Biological gradient**: Greater exposure should generally lead to greater incidence of the effect. However, in some cases, the mere presence of the factor can trigger the effect. In other cases, an inverse proportion is observed: greater exposure leads to lower incidence.
6. **Biological plausibility**: A plausible mechanism between cause and effect is helpful (but Hill noted that knowledge of the mechanism is limited by current knowledge).
7. **Coherence**: Consistency with the known natural history and biology of the disease supports causation.
8. **Experiment**: "Occasionally it is possible to appeal to experimental evidence".
9. **Analogy**: The presence of an analogous drug-disease causal relationship supports a causal inference.

I have employed weight-of-the-evidence[4] and adverse outcome pathway analyses[5] in this report. This approach is consistent with various guidance documents provided by the those of the United States Environmental Protection Agency (USEPA) and Organisation for Economic Co-operation and Development (OECD) Developmental Neurotoxicity (DNT) Guidelines (OCSPP 870.6300 or OECD TG 426)[6] as well as OECD TG 443 (OECD, 2018)[7] which require testing of learning and memory, and OECD

[4] Weight-of-the-evidence means the review of clinical observations, case reports, epidemiological and animal studies, toxicological experiments, exposure data, industrial hygiene and engineering studies, genetic testing, radiological findings, biochemical experiments, embryological information, medical diagnoses, and similar information that bear upon questions of causation. The manner in which scientists go about evaluating this data, including the assessment of the internal and external validity of this data is called a weight-of-the-evidence approach.

[5] Adverse outcome pathway analyses consider data describing the adverse consequences of exposure to a toxin at the molecular, cellular, tissue, organ, whole body, and population levels in assessing questions of association and causality.

[6] Health Effects Test Guidelines, OPPTS 870.6300, Developmental Neurotoxicity Study

[7] No. 151 Guidance Document Supporting OECD Test Guideline 443 on the Extended One-Generation Reproductive Toxicity Test

GD 43 (2008)[8] on reproductive toxicity, and ICH S5(R3) Detection of Reproductive and Developmental Toxicity for Human Pharmaceutical (ICH 2020), S5(R3) Detection of Reproductive and Developmental Toxicity for Human Pharmaceuticals (FDA 2021), and Reproductive and Developmental Toxicities-Integrating Study Results to Assess Concerns (FDA 2011). I have also been informed by Teratology (Wilson's Principles),[9] Weight of the Evidence, and systematic review methodology regarding proof of teratogenicity, developmental and reproductive toxicology, neurotoxicity, and establishment of causality.

Weight of Evidence:

The integration of animal and human data to assess the potential risk of medication use in pregnant women is necessary for several reasons. The foremost barrier for testing directly in pregnant women, is the risk of toxicity to the unborn child that could result in injury, malformations, or death. This risk is mitigated by performing reproductive and developmental toxicity testing on rodent and non-rodent animals, often referred to as preclinical testing. The second line of evidence is from human epidemiological studies, but these data are only made available after a medication has been approved for use and is then used by pregnant women. Study quality assessments of both human and animal data are incorporated for the weight of evidence (WoE) analysis.[10]

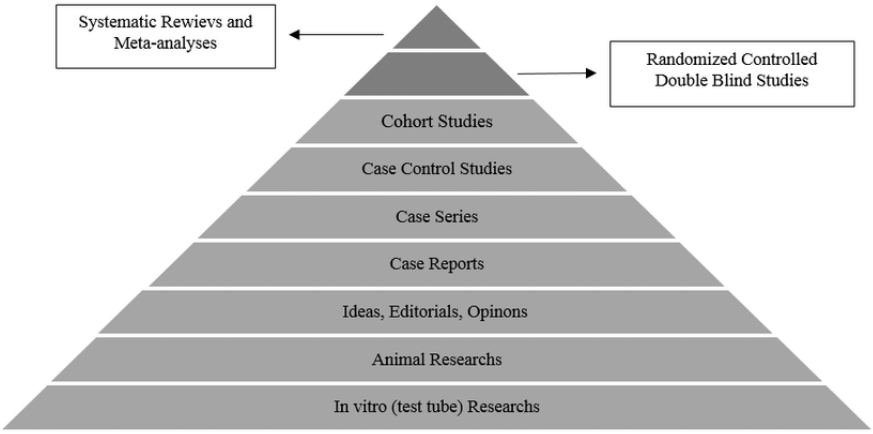

Figure 1. Hierarchy of Evidence.[11] The hierarchy of evidence categorizes studies to provide levels of evidence. It is used to classify the relevance of evidence to human health benefits or risks. The relevance is proposed to increase and potential for bias decrease at higher levels.

1. Animal Studies

Animal-based studies of developmental toxicology provide initial, generally accepted guidelines as to whether a compound, including a pharmaceutical drugor chemical, may present a teratogenic risk during pregnancy. The current testing guidelines provided by the FDA for reproductive toxicity rely upon the use of animal models to evaluate teratogenicity. Such testing procedures have the dual role of potentially identifying compounds that can interfere with normal development and elucidating the mechanism by

[8] No. 43 Guidance Document on Mammalian Reproductive Toxicity Testing and Assessment.
[9] The founding principles of the modern study of teratogens were initially articulated by James G. Wilson (1973), co-founder of The Teratology Society, in his monograph Environment and Birth Defects.
[10] Wurst et al. A model for human and animal data integration: Weight of evidence strategy. Birth Defects Res. 2020 Nov;112(18):1505-1512. doi: 10.1002/bdr2.1775. Epub 2020 Aug 7. PMID: 32770662.
[11] Reviewed in Murad MH, Asi N, Alsawas M, Alahdab F. New evidence pyramid. Evid Based Med. 2016 Aug;21(4):125-7. doi: 10.1136/ebmed-2016-110401. Epub 2016 Jun 23. PMID: 27339128; PMCID: PMC4975798; Figure from Evidence Based Practices in Health Sciences. ResearchGate. ISBN 978-954-07-4344-8.

which the dysmorphogenic events occur.

Using animal models is critically important in establishing the biological plausibility that a suspected compound could be a human teratogen. The physiological systems of mammals depend on common and conserved genetic, cellular, and tissue processes that havebeen shared and refined over time. Thus, the fundamental biology of how cells behave is shared among closely related species, allowing us to predict from animal studies what is expected to occur in humans.[12]

The International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) has set forth generally accepted guidelines for testing the teratogenicity of a compound.[13] This testing is generally performed in two species, rabbits and rats. Additionally, mice are an acceptablealternative testing model that is used in place of rats. Tests using mice produce quality study models equally as predictive as rat designs. Moreover, as the mouse genome is comparable to humans, both in size and complexity, mice are very well suited for studies that mimic teratogenicexposures in humans.

Animal testing proceeds by exposing a pregnant animal (dam) to a test compound by an appropriate route of administration during a selected period of gestation. Some studies are restricted to the period of organogenesis, when the major organ systems of theembryo are forming. In humans, with the caveat that the date of "conception" and last menstrual period may be unclear in an individual female, weeks 2-8 post-conception were considered the period of organogenesis, although more recent terminology refers to the first eight weeks post-conception as the Embryonic Period.[14] In rodents, typically days 6-12 of gestation are tested, which encompass the major events of organogenesis. Other experimental paradigms might have the treatment start at fertilization or implantation, the time when the fertilized egg, now a blastocyst, attaches to the uterus, and the treatment is administered daily until the end of the selected study period.[15]

The physical examination of the soft tissues of embryos typically involves preparing histological sections of fixed fetuses,[16] although combinations of methods using fresh and fixed material are not uncommon.[15] For these studies, thefetal tissue is placed in fixative solution to cross-link proteins and thereby stiffen it so that it can be sliced by a razor blade and the thick pieces (sections) are examined under a microscope. To resolve pathology at a more sensitive level, the tissue is fixed, embedded in paraffin wax, and then thin (micrometer) sections are cut on a microtome. The terminology usedto describe the specific defects observed in the sectioned fetal tissue has been standardized to be descriptive, rather than diagnostic or interpretative.[17] The data generated from each study animal can be tabulated for statistical analyses,

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[12] Daston. Laboratory models and their role in assessing teratogenesis. Am J Med Genet C Semin Med Genet. 2011 Aug 15;157C(3):183-7. doi: 10.1002/ajmg.c.30312. Epub 2011 Jul 15. PMID: 21766439.

[13] Cavagnaro. Preclinical safety evaluation of biotechnology-derived pharmaceuticals. Nat Rev Drug Discov. 2002 Jun;1(6):469-75. doi: 10.1038/nrd822. PMID: 12119749.

[14] Sadler and Langman (2012). Langman's medical embryology. Philadelphia, Wolters Kluwer Health/Lippincott Williams & Wilkins.

[15] Wise et al. Embryo-fetal developmental toxicity study design for pharmaceuticals. Birth Defects Res B Dev Reprod Toxicol. 2009 Dec;86(6):418-28. doi: 10.1002/bdrb.20214. PMID: 20025038.

[16] Wilson. (1973). Environment and birth defects. New York,, Academic Press.

[17] Makris et al. Terminology of developmental abnormalities in common laboratory mammals (version 2). Reprod Toxicol. 2009 Nov;28(3):371-434. doi: 10.1016/j.reprotox.2009.06.010. Epub 2009 Sep 1. Erratum in: Reprod Toxicol. 2012 Nov;34(3):487. PMID: 19729062.

including data on: pre-implantation loss (the death of embryos from the time they are conceived until just before they implant in the uterus), resorptions (fetuses that implanted, developed for some time, then died and all that remains is a small clot of disorganized tissue), dead fetuses, live fetuses, fetal weight and the fetal evaluations. The analyses should make comparisons to the concurrent control group, although it can be useful to refer back to historical control data at times.[15] In assessing the weight-of-the-evidence for each animal study, I employed the six Quality Assessment Point for animal studies:

The Six Quality Assessment Points to be Evaluated in Animal Studies:[18]

Animal data	
Point to evaluate	**Areas of evaluation**
Test article	• Source • Purity • Vehicle • Evidence of accurate formulation • Evidence of stability appropriate for study
Test system	• Species • Gender • Age range • Husbandry information
Control group	• Vehicle and/or positive control
Study design or purpose	• Number of animals • Route of administration • Exposure information including dosage and duration • Statistical methods
Endpoints	• If appropriate for evaluation of study objective • If not standard, rationale provided
Results	• Presented transparently • Clear/correct identification of test article-related effects • Conclusions are reasonable based on the study design and results

[18] Wurst et al. A model for human and animal data integration: Weight of evidence strategy. Birth Defects Res. 2020 Nov;112(18):1505-1512. doi: 10.1002/bdr2.1775. Epub 2020 Aug 7. PMID: 32770662.

2. Human Epidemiological Evidence

The field of epidemiology plays a vital role in determining theteratogenicity of a pharmaceutical compound. The study of the occurrence of illness and disease in human populations, especially the cause(s) and determinants of patterns of disease, is a criticalcomponent to my work as a teratologist. Toxicological experiments are generally not conducted in pregnant women for obvious ethical reasons; therefore, teratologists often rely upon epidemiological studies of pregnancy outcomes related to maternal drug exposure to analyze human teratogenicity. Pertinent epidemiological studies, coupled with the animal experiments and application of Wilson's principles, inform any conclusion related to the teratogenicity of a medication. As such, teratologists must interpret and rely upon clinical case reports, drug pregnancy registries, prospective cohorts, retrospective cohorts, adverse event reporting, exposure monitoring systems, post-marketing surveillance, and epidemiological studies,including meta-analyses, to help inform their decisions concerning the teratogenic nature of a drug in the human situation. In assessing the weight-of-the-evidence for each epidemiological study, I employed these six Quality Assessment Points:

The Six Quality Assessment Points to be Evaluated in Human Studies:[18]

Human data	
Point to evaluate	**Areas of evaluation**
Study design or purpose	• Type of design • If study is appropriate for the question of interest • If question aims to generate versus test a hypothesis
Exposure classification	• If information is available about medication frequency, dose, and duration of use • If exposure is from an appropriate time window • If exposure is prospectively collected • If exposure is collected from an automated source • If exposure is validated
Outcome classification	• Outcome definition • Outcome ascertainment • Outcome diagnosis criteria • If outcome is validated
Control group	• If there is an internal control group • If control group is disease-based
Study size	• If power calculations are conducted • If confidence limits are provided • Potential for multiple testing
Confounding factors or biases	• If confounders controlled for • If additional biases accounted for and reduced

3. Systematic Reviews and Meta-Analyses

Hierarchy of Evidence, Level 1

These are comprehensive studies that combine and analyze the results of multiple research studies. In the Hierarchy of Evidence, systematic reviews and meta-analyses are at the top of the hierarchy. They are the highest levels of evidence because they provide a comprehensive and rigorous analysis of multiple studies on a particular topic.

A systematic review is a methodical way of examining all the scientific studies related to a specific topic or question. Researchers carefully select and analyze relevant studies that meet certain criteria to create an unbiased and complete summary of the evidence. The goal is to provide a clear answer to the research question based on all available information.

Within a systematic review, researchers often use a technique called meta-analysis. A meta-analysis combines the results from multiple studies, pooling their data together to obtain a more precise estimate of the treatment effect or association. This statistical approach allows researchers to analyze the combined evidence, providing a more comprehensive and reliable summary of the overall findings.

By combining a systematic review with a meta-analysis, researchers can evaluate the evidence more effectively. This helps them estimate the overall impact of a treatment or association more accurately and draw more reliable conclusions. Systematic reviews and meta-analyses are important tools used in evidence-based practice, decision-making, and shaping healthcare policies and guidelines.

Adverse Outcome Pathway: Framework for Analysis of Acetaminophen Exposures

Adverse Outcome Pathway (AOP) analysis is a framework used by various regulatory organizations to understand the causal relationship between chemical exposures and adverse effects on living organisms. AOPs are structured frameworks that provide a clear and transparent understanding of the biological events that lead to a specific adverse outcome. The process involves identifying key biological events, termed key events (KEs), and the relationships between them (links), leading to the adverse outcome (AO). By identifying the KEs involved in the pathway leading to AOs, AOPs provide a widely accepted foundation to evaluate the potential toxicity of chemicals. This approach is summarized in the AOP Fact Sheet (Figure 2).

Regulatory organizations use AOP analysis to support a variety of decision-making processes, including chemical prioritization, hazard identification, and risk assessment. The use of AOPs has become increasingly important as new chemicals and chemical mixtures are introduced into the environment. By providing a standardized framework for understanding the potential adverse outcomes associated with chemical exposures, AOPs support the development of effective risk management strategies that protect public health and the environment.

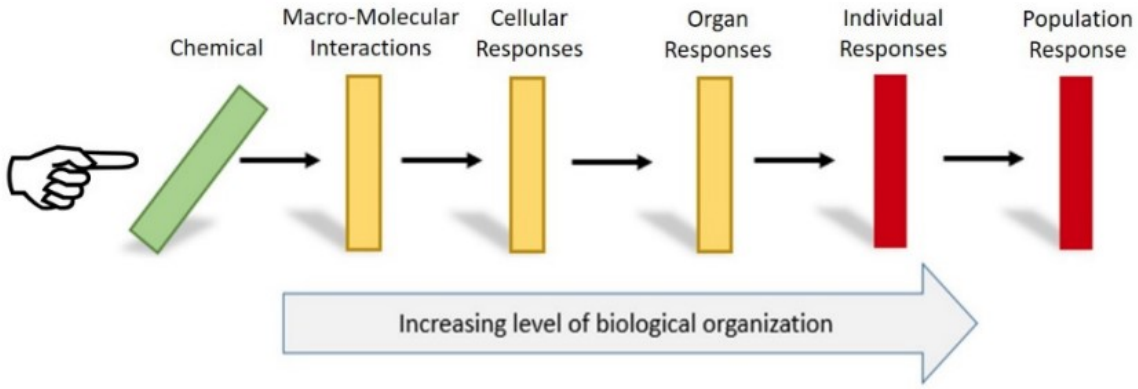

Figure 2. Adverse Outcome Pathway (AOP).[19] AOPs map how stressors interact within an organism to cause adverse outcomes.

[19] AOP Fact Sheet – US Environmental Protection Agency (US EPA).

BACKGROUND: ACETAMINOPHEN

Acetaminophen is an organic compound with a molecular formula of C8H9NO2 and a molecular weight of 151.16 g (CAS # 103-90-2). It is an acetyl derivative of p-aminophenol/4-aminophenol (Figure 3) and is also known as N-acetyl-p-aminophenol (APAP), paracetamol, or 4-acetamidophenol, and pharmaceutical brand names include Tylenol (J&J) and Panadol (GSK). While limited APAP synthesis and usage was reported in the 1800s, the Tylenol brand was first introduced and gained a wide market in 1955, by McNeil Laboratories, as a pain-relief/analgesic and fever-reducing/anti-pyretic medication for children. Johnson & Johnson acquired McNeil Laboratories in 1959 and began marketing Tylenol over the counter (OTC) and towards adults by 1961. Marketed as a safe alternative to aspirin, Johnson & Johnson reports Tylenol as one of the company's most widely used and iconic products.[20]

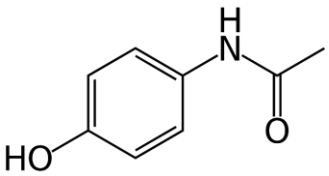

Figure 3. Chemical Structure of Acetaminophen (APAP)

The Aniline Analgesics

Acetanilide, phenacetin, and paracetamol (acetaminophen, APAP) are the original members of the drug class referred to as aniline analgesics. Acetanilide was the first of these molecules to gain usage as an antipyretic, when it was reported in 1886 that it was mistakenly used in place of naphthalene to treat intestinal worms and resulted in a reduction in fever.[21] The use of acetanilide was limited due to toxicity resulting in methemoglobinemia and cyanosis. As the use of acetanilide was limited by toxicity, the search for other aniline derivatives resulted in the introduction of phenacetin and paracetamol. Paracetamol was initially overlooked due to an assumption that phenacetin was less toxic, so phenacetin was the predominate aniline analgesic. Phenacetin usage continued for several decades, but usage gradually fell after it was reported in 1948, that metabolites of acetanilide included aniline and paracetamol.[22] Phenacetin was ultimately withdrawn from the market in the 1970s and 1980s, with reports of a high potential for misuse and an unfavorable benefit-to-risk ratio.[23] There were also reports in the 1960s, that phenacetin was associated with renal lesions and nephrotoxicity.[24] Phenacetin was eventually classified by the International Agency for Research on Cancer (IARC) in 1987 as probably carcinogenic to humans (Group 2A) and analgesic mixtures containing phenacetin as carcinogenic (Group 1).[25] While APAP is promoted as one of the most popular and safe pain-relief medications, **APAP toxicity is responsible for almost**

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[20] Company History, Johnson & Johnson, https://ourstory.jnj.com/timeline

[21] Brune et al. Acetaminophen/paracetamol: A history of errors, failures and false decisions. Eur J Pain. 2015 Aug;19(7):953-65. doi: 10.1002/ejp.621. Epub 2014 Nov 27. PMID: 25429980.

[22] Brodie and Axlerod. The estimation of acetanilide and its metabolic products, aniline, N-acetyl p-aminophenol and p-aminophenol, free and total conjugated, in biological fluids and tissues. J Pharmacol Exp Ther. 1948 Sep;94(1):22-8. PMID: 18885610.

[23] FDA, Federal Register, Vol 48, No 194, Wednesday, October 5, 1983.

[24] Tan et al. Is phenacetin a nephrotoxin? a report on twenty-three users of the drug. Calif Med. 1964 Aug;101(2):73-7. PMID: 14180501; PMCID: PMC1515485.

[25] IARC: Overall evaluations of carcinogenicity: an updating of IARC monographs volumes 1 to 42. 1987, Lyon: International Agency for Research on Cancer, 7:
https://monographs.iarc.who.int/wp-content/uploads/2018/06/Suppl7-121.pdf

half (46%) of all acute liver failure cases in the United States, and it is estimated that half of these cases are unintentional.[26,27]

1. Acetaminophen and Aniline

Acetaminophen remains as the only aniline analgesic still in use today, and as explained below, because APAP is a metabolite of aniline, the wide-spread use of industrial aniline in the environment results in nearly universal human exposure to APAP, although at very low levels.

The other members of the aniline analgesic class have been banned for clinical use, but aniline remains an important source material in various chemical industries (e.g., rubber, pesticides, and pharmaceuticals). An IMARC market overview indicates the global aniline market size reached 9.4 million tons in 2022.[28] As indicated below, industrialized population are reported to be ubiquitously exposed to aniline, and thereby APAP as a metabolite of aniline.

A European Chemicals Bureau risk assessment report has estimated that the human exposure to aniline from fruit and vegetables is approximately 0.11 mg/kg bodyweight per day, while humans near industrial sources have an exposure burden estimated at 0.74 mg/kg/day.[29] Biological monitoring in Germany reported ubiquitous urinary excretion of APAP in the general population and examined possible sources: (1) direct intake of paracetamol through medication, (2) paracetamol residues in the food chain and (3) environmental exposure to aniline or related substances that are metabolized into APAP.[30] The proposed conversion of aniline to APAP was tested in an animal model, and aniline was shown to be converted nearly completely in the liver of the mouse to APAP.[31] Aniline conversion to APAP was then tested in four healthy male volunteers with a 5 mg dose of isotope-labeled aniline, and APAP was the predominant urinary aniline metabolite representing 55.7-68.9 % of the oral dose.[32]

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[26] Ramachandran A, Jaeschke H. Acetaminophen Hepatotoxicity. Semin Liver Dis. 2019 May;39(2):221-234. doi: 10.1055/s-0039-1679919. Epub 2019 Mar 8. PMID: 30849782; PMCID: PMC6800176.

[27] Agrawal and Khazaeni. Acetaminophen Toxicity. [Updated 2023 Feb 12]. In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing; 2023 Jan-. Available from: https://www.ncbi.nlm.nih.gov/books/NBK441917/

[28] Aniline Market Size, Share, Price Trends, Analysis, 2023-2028 (imarcgroup.com): https://www.imarcgroup.com/aniline-market

[29] EU Summary risk assessment report (europa.eu): https://echa.europa.eu/documents/10162/462b7066-c639-4883-b384-3daf4ec88ded

[30] Dierkes G, et al. N-Acetyl-4-aminophenol (paracetamol), N-acetyl-2-aminophenol and acetanilide in urine samples from the general population, individuals exposed to aniline and paracetamol users. Int J Hyg Environ Health. 2014 Apr-May;217(4-5):592-9. doi: 10.1016/j.ijheh.2013.11.005. Epub 2013 Dec 6. PMID: 24370547.

[31] Holm JB, et al. Aniline Is Rapidly Converted Into Paracetamol Impairing Male Reproductive Development. Toxicol Sci. 2015 Nov;148(1):288-98. doi: 10.1093/toxsci/kfv179. Epub 2015 Aug 10. PMID: 26259604.

[32] Modick H, et al. Human metabolism and excretion kinetics of aniline after a single oral dose. Arch Toxicol. 2016 Jun;90(6):1325-33. doi: 10.1007/s00204-015-1566-x. Epub 2015 Aug 2. PMID: 26233686.

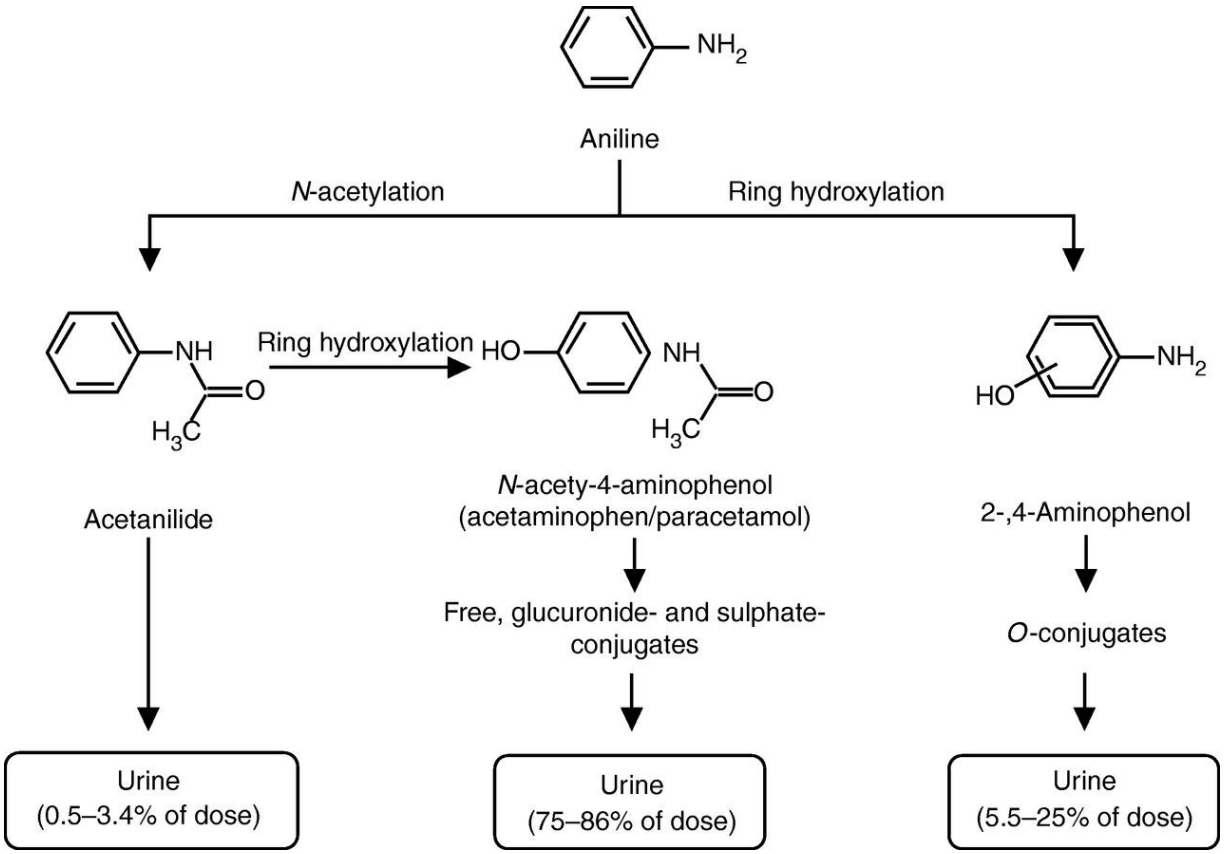

Figure 4. Simplified Metabolism of Aniline to Acetaminophen (APAP).[33]

Ubiquitous environmental exposures to the industrial chemical aniline results in conversion to APAP in the liver of laboratory animals and humans, resulting in a low-level chronic exposure to and excretion of APAP (Figure 4). The pharmaceutical consumption of APAP increases this exposure above background level, producing environmental (background) versus pharmaceutical exposures that differ by 1000-fold (Figure 5).

[33] Modick H, et al. Ubiquitous presence of paracetamol in human urine: sources and implications. Reproduction. 2014 Mar 4;147(4):R105-17. doi: 10.1530/REP-13-0527. PMID: 24451225.

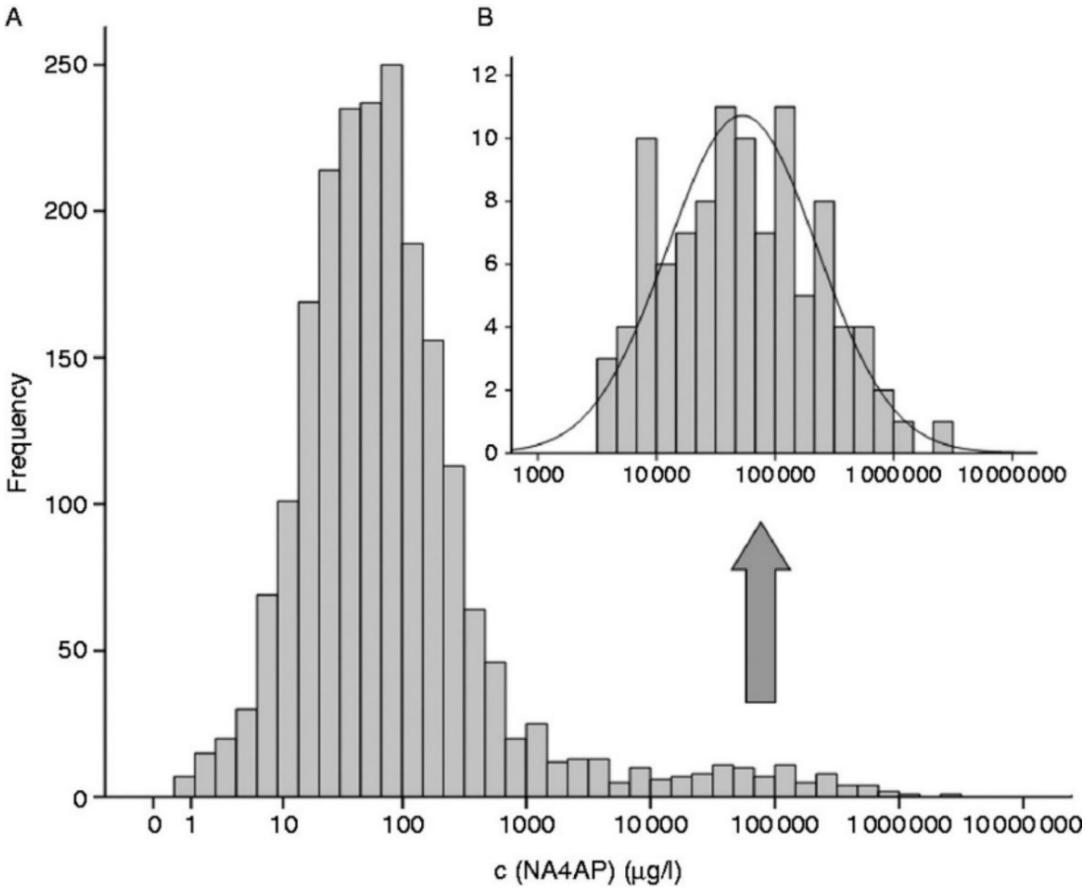

Figure 5. Distribution of Urinary APAP. Concentrations in random spot urine samples obtained from the general population: (A) all samples (n=2098) and (B) samples of the high-exposure group (n=106; c>4000 µg/l). The high exposures are due to pharmacological intake of APAP. Line indicates normal distribution curve.[34]

This study showed that the general population is exposed to APAP at a relatively low level, with a median exposure of about 80-100 micrograms/L, and this exposure is proposed to originate from the metabolism of the industrial chemical aniline into APAP. These low concentrations contrast with the concentrations found in people reported to have recently taken APAP as a pharmaceutical (Figure 5, B). Pharmaceutical APAP intake results in increased urinary APAP to approximately 1,000 times greater than the general population. Accordingly, APAP is expected to be detected in the general population, even if this population has not taken or reported recent use of APAP, and this background level of APAP is reported to be due to environmental exposure to aniline and conversion of it into APAP. **This review of aniline conversion into APAP is provided to explain the reported detection of APAP in human biologics, even in the absence of reported pharmaceutical intake of APAP.**

Acetaminophen Metabolism

Acetaminophen is a weak organic acid with moderate lipid-solubility that allows passive diffusion across the small intestine and cellular membranes. The absorption from the gastrointestinal tract is dose-

[34] Modick H, et al. Ubiquitous presence of paracetamol in human urine: sources and implications. Reproduction. 2014 Mar 4;147(4):R105-17. doi: 10.1530/REP-13-0527. PMID: 24451225.

dependent, and ranges from 70-90%.[35] The Cmax was reported between 30-90 minutes when given to 144 subjects, with a mean concentration of 16 microgram/mL in whole blood when subjects were given a dose of 1000mg.[36] Once absorbed, the metabolism of acetaminophen has been well documented for decades (Figure 6). Briefly, about 50-70% and 25-35% of a therapeutic dose is metabolized and excreted in the urine as glucuronic and sulfuric acid conjugates, respectively, whereas the toxic-reactive metabolite N-acetyl-p-benzoquinone imine (NAPQI), gives rise to mercapturic acid and cysteine conjugates and accounts for 5-15% of the dose.[37]

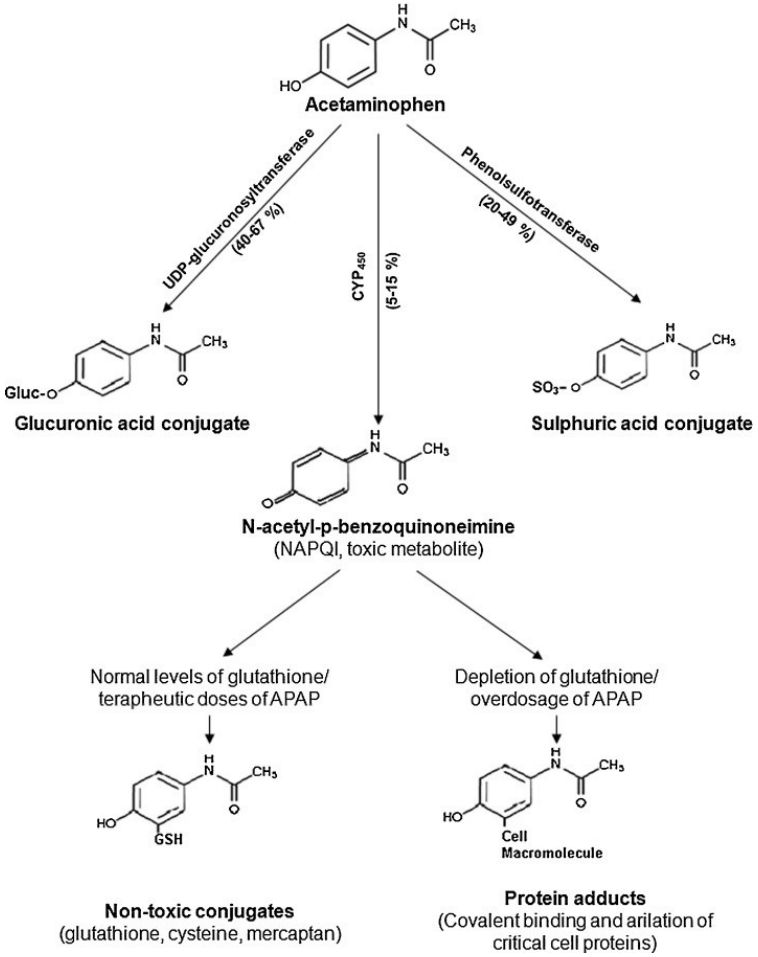

Figure 6. Liver Acetaminophen (APAP) Metabolism. There are three main metabolic pathways for APAP in liver after administration of therapeutic or toxic doses.[38]

[35] Forrest et al. Clinical pharmacokinetics of paracetamol. Clin Pharmacokinet. 1982 Mar-Apr;7(2):93-107. doi: 10.2165/00003088-198207020-00001. PMID: 7039926.

[36] Gwilt et al. The absorption characteristics of paracetamol tablets in man. J Pharm Pharmacol. 1963 Jul;15:445-53. doi: 10.1111/j.2042-7158.1963.tb12812.x. PMID: 13951544.

[37] Prescott LF. Kinetics and metabolism of paracetamol and phenacetin. Br J Clin Pharmacol. 1980 Oct;10 Suppl 2(Suppl 2):291S-298S. doi: 10.1111/j.1365-2125.1980.tb01812.x. PMID: 7002186; PMCID: PMC1430174.

[38] Ghanem et al. Acetaminophen from liver to brain: New insights into drug pharmacological action and toxicity. Pharmacol Res. 2016 Jul;109:119-31. doi: 10.1016/j.phrs.2016.02.020. Epub 2016 Feb 26. PMID: 26921661; PMCID: PMC4912877.

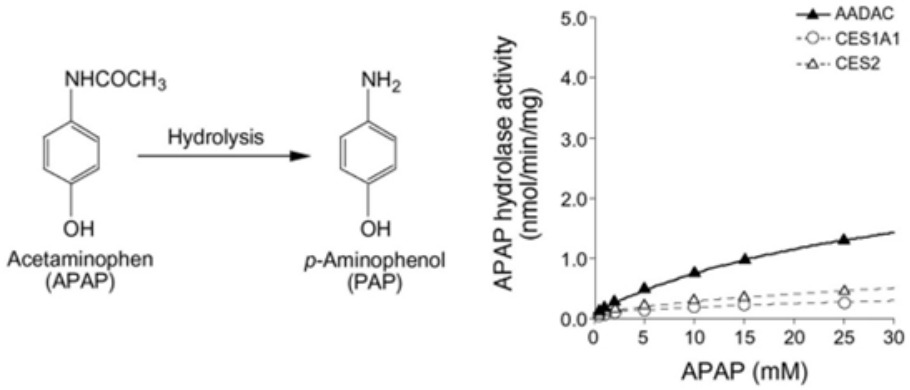

Figure 7. Acetaminophen (APAP) Hydrolysis. (Left) The hydrolysis of APAP into PAP. (Right) The relative activity of carboxylesterases, CES1A1 and CES2, and arylacetamide deacetylase (AADAC) on APAP hydrolysis were reported in 2010.[39]

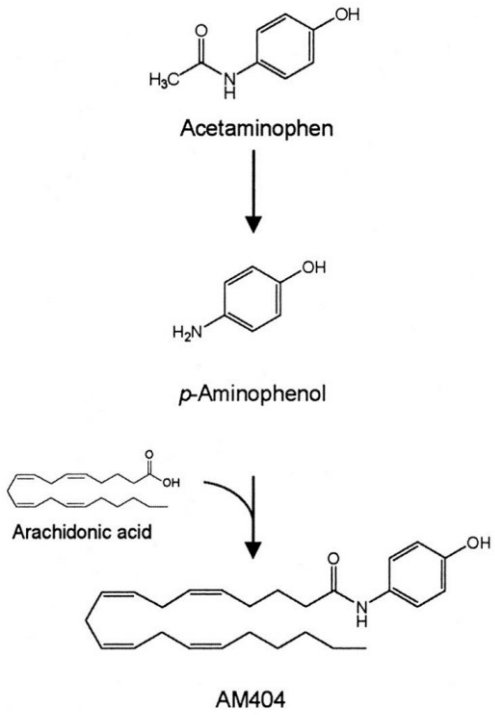

Figure 8. Acetaminophen (APAP) Hydrolysis, Arachidonic Acid Conjugation, and AM404.[40]

The dependence of AM404 formation from PAP by fatty acid amide hydrolase (FAAH) was reported in 2005.[40] APAP can also be metabolized via hydrolysis by AADAC (Figure 7). This process is the first step in arachidonic acid conjugation of PAP by FAAH, forming AM404 (Figure 8). FAAH is found in various

[39] Watanabe et al. Arylacetamide deacetylase is a determinant enzyme for the difference in hydrolase activities of phenacetin and acetaminophen. Drug Metab Dispos. 2010 Sep;38(9):1532-7. doi: 10.1124/dmd.110.033720. Epub 2010 Jun 11. PMID: 20542992.

[40] Högestätt et al. Conversion of acetaminophen to the bioactive N-acylphenolamine AM404 via fatty acid amide hydrolase-dependent arachidonic acid conjugation in the nervous system. J Biol Chem. 2005 Sep 9;280(36):31405-12. doi: 10.1074/jbc.M501489200. Epub 2005 Jun 29. PMID: 15987694.

tissues, including liver, thyroid, testes, and brain, with high expression in the dentate gyrus, hippocampus, orbital cortex, primary motor and primary somatosensory cortex.[41]

Pharmaceutical Mechanisms of Action for Acetaminophen

The indications of acetaminophen include headache, pain, and fever. The suggested therapeutic dose of acetaminophen for adults is two tablets of 325mg, 500 mg, or 650mg, taken orally every 4-8 hours, with a maximum of 4 grams for any 24-hour period. Despite decades of use, the mechanism of action for acetaminophen has not been precisely quantified. There are currently multiple mechanisms of action proposed and numerous questions remain regarding the relative impact of each proposed mechanism on biological impacts (Figure 9).

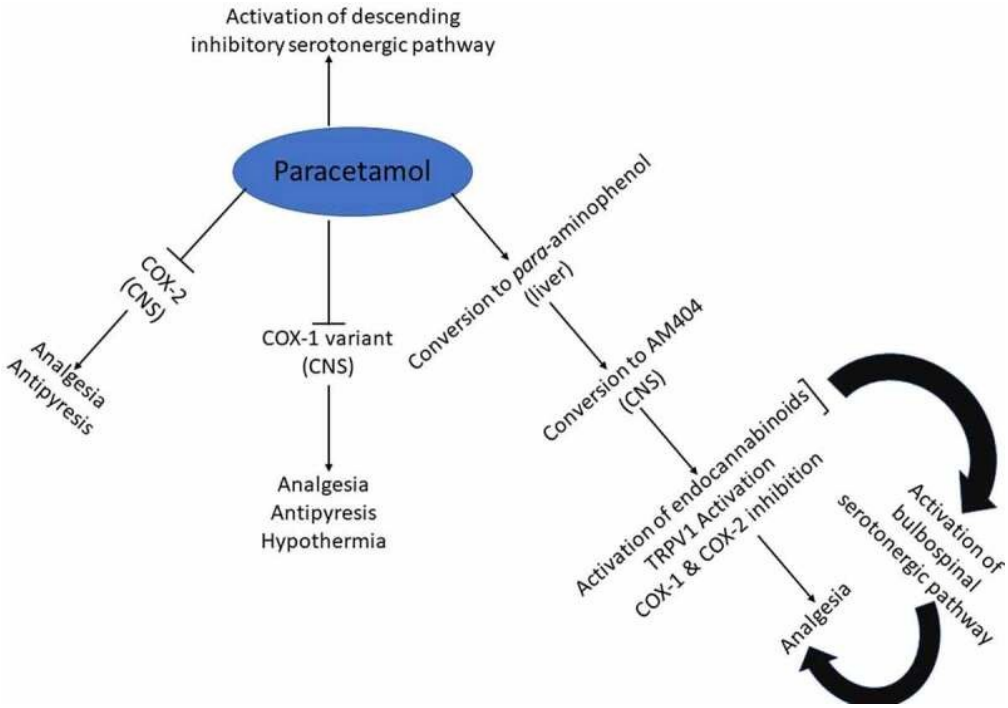

Figure 9. Schematic Representation of Proposed Pharmacological Mechanisms of Action of Paracetamol.[42]

1. COX Interactions

The proposed mechanisms of actions are based on activities in the central nervous system (CNS), where APAP is reported to increase pain threshold and thereby cause pain relief. One of the first proposed mechanisms of the analgesic action of APAP was the inhibition of cyclooxygenase enzymes (COX-1 and

[41] Egertováet al. Comparative analysis of fatty acid amide hydrolase and cb(1) cannabinoid receptor expression in the mouse brain: evidence of a widespread role for fatty acid amide hydrolase in regulation of endocannabinoid signaling. Neuroscience. 2003;119(2):481-96. doi: 10.1016/s0306-4522(03)00145-3. PMID: 12770562.

[42] Ayoub SS. Paracetamol (acetaminophen): A familiar drug with an unexplained mechanism of action. Temperature (Austin). 2021 Mar 16;8(4):351-371. doi: 10.1080/23328940.2021.1886392. PMID: 34901318; PMCID: PMC8654482.

COX-2), which are involved in prostaglandin (PG) synthesis.[43,44] Additional studies have also introduced the potential of inhibition of a splice variant of COX-1, referred to as COX-1b or COX-3 in various publication.[45,46]

2. Serotonergic Interactions

In addition to COX inhibition, activation of the descending inhibitory serotonergic pathway by APAP has also been proposed to provide analgesic action in animal models and human. The serotonergic receptors implicated in these studies include 5-HT1A,[47] 5-HT3,[48] and 5-HT7.[49] In addition, depletion of serotonin (5-HT), by the administration of p-chlorophenylalanine, was shown to attenuate the analgesic action of APAP,[50] but the influence of APAP on serotonergic levels and signaling is proposed to be indirect (Figure 10), as APAP has not shown a relevant affinity for any of the serotonin receptors or transporter.[51]

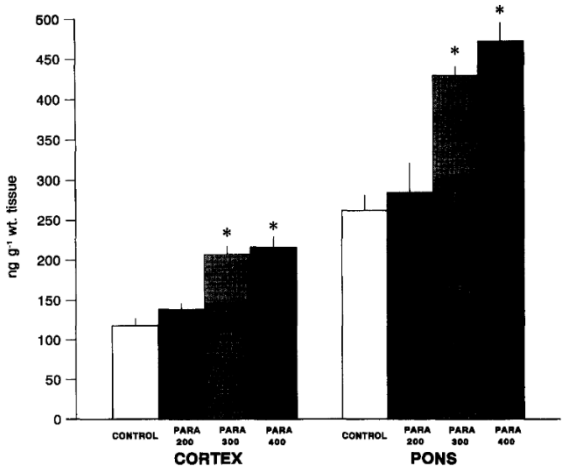

Figure 10. Impact of Acetaminophen (APAP/PARA) at 200, 300, and 400mg/Kg on Serotonin (5-HT ng/g tissue) in Cortical (CORTEX) and Pontine (PONS) Brain Areas. Values are average +/- SEM for N=6 rats per group, *

[43] Flower and Vane. Inhibition of prostaglandin synthetase in brain explains the anti-pyretic activity of paracetamol (4-acetamidophenol). Nature. 1972 Dec 15;240(5381):410-1. doi: 10.1038/240410a0. PMID: 4564318.
[44] Mitchell et al. Selectivity of nonsteroidal antiinflammatory drugs as inhibitors of constitutive and inducible cyclooxygenase. Proc Natl Acad Sci U S A. 1993 Dec 15;90(24):11693-7. doi: 10.1073/pnas.90.24.11693. PMID: 8265610; PMCID: PMC48050.
[45] Willoughby, et al. COX-1, COX-2, and COX-3 and the future treatment of chronic inflammatory disease. Lancet. 2000 Feb 19;355(9204):646-8. doi: 10.1016/S0140-6736(99)12031-2. PMID: 10696997.
[46] Chandrasekharan et al. COX-3, a cyclooxygenase-1 variant inhibited by acetaminophen and other analgesic/antipyretic drugs: cloning, structure, and expression. Proc Natl Acad Sci U S A. 2002 Oct 15;99(21):13926-31. doi: 10.1073/pnas.162468699. Epub 2002 Sep 19. PMID: 12242329; PMCID: PMC129799.
[47] Karandikar et al. Effect of drugs modulating serotonergic system on the analgesic action of paracetamol in mice. Indian J Pharmacol. 2016 May-Jun;48(3):281-5. doi: 10.4103/0253-7613.182874. PMID: 27298498; PMCID: PMC4900001.
[48] Pelissier et al. Paracetamol exerts a spinal antinociceptive effect involving an indirect interaction with 5-hydroxytryptamine3 receptors: in vivo and in vitro evidence. J Pharmacol Exp Ther. 1996 Jul;278(1):8-14. PMID: 8764329.
[49] Dogrul et al. Systemic paracetamol-induced analgesic and antihyperalgesic effects through activation of descending serotonergic pathways involving spinal 5-HT$_7$ receptors. Eur J Pharmacol. 2012 Feb 29;677(1-3):93-101. doi: 10.1016/j.ejphar.2011.12.016. Epub 2011 Dec 21. PMID: 22206817.
[50] Pini et al. The antinociceptive action of paracetamol is associated with changes in the serotonergic system in the rat brain. Eur J Pharmacol. 1996 Jul 11;308(1):31-40. doi: 10.1016/0014-2999(96)00261-0. PMID: 8836629.
[51] Raffa and Codd. Lack of binding of acetaminophen to 5-HT receptor or uptake sites (or eleven other binding/uptake assays). Life Sci. 1996;59(2):PL37-40. doi: 10.1016/0024-3205(96)00273-1. PMID: 8699917.

$P < 0.05$ vs. control values (ANOVA followed by Student-Newman-Keuls' test).[52] This 5-HT response by APAP has also been reported in the frontal cortex and brainstem.[53]

While APAP has not been shown to have a high affinity for serotonin receptors or the serotonin transporter (SERT), the activity of APAP on pain (antinociceptive) has been shown to be dependent in part on the ability of APAP to modify serotonin concentrations. These indirect serotonergic interactions have been reported in multiple studies. Regarding direct interactions, at 10 μM, APAP inhibited less than 10% of radioligand binding to serotonin receptors or transporter.[51] Regarding indirect interactions, the involvement of serotonin receptors, 5-HT(1A/B), in the antinociceptive effect of APAP was demonstrated by modifying the interactions of known antagonists of 5-HT1A and 5-HT1B in mice.[54] Similar results have also been reported in rats, indicating that APAP modifies the interaction between serotonin and the 5-HT1B receptor.[55,56] Loss of 5-HT1B is reported to alter behavior in mice including altered impulse control, behavioral disinhibition, and stimulus specific hyperreactivity.[57,58] The ability of APAP to modify serotonin and interactions with 5-HT1B has CNS and vascular impacts. For example, 5-HT1b also mediates vasoconstriction in human umbilical arteries.[59,60,61]

The influence of serotonin on vascular function, and specifically the biphasic dose response of 5-HT on human umbilical arteries has been observed in numerous studies.[62,63,64] Moreover, the naming and function of serotonin is actually based on its activity as a serum vasoconstrictor.[65] Excess 5-HT is also a demonstrated teratogen in various animal models, capable of producing a wide-spectrum of congenital

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[52] Pini et al. The antinociceptive action of paracetamol is associated with changes in the serotonergic system in the rat brain. Eur J Pharmacol. 1996 Jul 11;308(1):31-40. doi: 10.1016/0014-2999(96)00261-0. PMID: 8836629.

[53] Vijayakaran K, et al. Arsenic decreases antinociceptive activity of paracetamol: possible involvement of serotonergic and endocannabinoid receptors. Environ Toxicol Pharmacol. 2014 Sep;38(2):397-405. doi: 10.1016/j.etap.2014.07.001. Epub 2014 Jul 10. PMID: 25128769.

[54] Roca-Vinardell et al. The role of 5-HT1A/B autoreceptors in the antinociceptive effect of systemic administration of acetaminophen. Anesthesiology. 2003 Mar;98(3):741-7. doi: 10.1097/00000542-200303000-00025. PMID: 12606921.

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[56] Sandrini et al. Differential involvement of central 5-HT1B and 5-HT3 receptor subtypes in the antinociceptive effect of paracetamol. Inflamm Res. 2003 Aug;52(8):347-52. doi: 10.1007/s00011-003-1185-5. PMID: 14504673.

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[62] Haugen and Moe. In vitro perfusion studies of single umbilical artery cords: the vasoactive effects of serotonin. Acta Obstet Gynecol Scand. 1999 Apr;78(4):285-9. PMID: 10203293.

[63] Haugen and Rognerud. Doppler flow velocity waveforms and vasoactive effects of serotonin in human umbilical arteries. Gynecol Obstet Invest. 2001;51(1):22-7. doi: 10.1159/000052885. PMID: 11150870.

[64] Haugen. Influence of gestational age on the vasodilatory response of serotonin in human umbilical arteries perfused in vitro. Gynecol Obstet Invest. 2008;66(2):98-103. doi: 10.1159/000128280. Epub 2008 Apr 28. PMID: 18441526.

[65] Rapport et al. Serum vasoconstrictor, serotonin; isolation and characterization. J Biol Chem. 1948 Dec;176(3):1243-51. PMID: 18100415.

malformations.[66,67,68] While APAP is reported to increase serotonin in the CNS, APAP is reported to inhibit serotonin release by platelets and reduce vasoconstriction.[69,70] The proposed mechanism for these divergent interactions is via differences in COX enzymes, as indicated above. Decreased plasma 5-HT due to pharmacological or genetic depletion has also been reported to increase the hepatotoxicity of APAP in mice.[71]

3. Cannabinoid Interactions

While the main metabolic routes for APAP in the liver have been studied for decades, in 2005 another APAP metabolite, p-aminophenol (PAP), was identified in the liver, blood, and brain, accounting for 1-2% of APAP metabolism,[72] and which is further conjugated with arachidonic acid to form the bioactive fatty acid amide N-arachidonoyl phenolamine (AM404) in the brain.[73] The conversion of PAP to AM404 was reported to be dependent on fatty acid amide hydrolase (FAAH), as mutant mice lacking FAAH did not produce the AM404 metabolite. AM404 was also reported to be a potent activator (nM) of TRPV1, a ligand at cannabinoid CB1 receptors and an inhibitor of cellular anandamide uptake, leading to increased levels of anandamide, an endogenous cannabinoid. Additional studies have reported that AM404 is also an inhibitor of the arachidonoylethanolamide (AEA) membrane transporter (AMT) and can activate the vanilloid (capsaicin) receptor (VR1) in the nM range.[74]

Anandamide is an endogenous modifier of cannabinoid signaling via cannabinoid CB1 receptor, and activation by endogenous or exogenous cannabinoids can produce a range of different impacts on learning, behavior, and development. In order to examine the role of loss of CB1, mutant mice were produced that had CB1 mutated. These animals displayed disruption of learning and behavior, specifically the animals were unable to unlearn bad memories, termed extinction of aversive memories.[75] This is based on laboratory testing of fear conditioning, such that a ringing tone or flash of light is coupled with pain, via a shock or other noxious exposure. After a few times, the animal learns to respond fearfully to the ringing tone or light. Extinction can then be performed by allowing the animal to hear the sound or see the light

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[66] Poulson et al. Teratogenic effect of 5-hydroxytryptamine in mice. Science. 1963 Aug 23;141(3582):717-8. doi: 10.1126/science.141.3582.717. PMID: 13985782.

[67] Reddy et al. Teratogenic effects of serotonin. J Pediatr. 1963 Sep;63:394-7. doi: 10.1016/s0022-3476(63)80425-4. PMID: 14061025.

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[71] Zhang et al. Serotonin deficiency exacerbates acetaminophen-induced liver toxicity in mice. Sci Rep. 2015 Jan 29;5:8098. doi: 10.1038/srep08098. Erratum in: Sci Rep. 2015;5:12184. PMID: 25631548; PMCID: PMC4309973.

[72] Nicholls et al. NMR and HPLC-NMR spectroscopic studies of futile deacetylation in paracetamol metabolites in rat and man. J Pharm Biomed Anal. 1997 Apr;15(7):901-10. doi: 10.1016/s0731-7085(96)01950-4. PMID: 9160256.

[73] Högestätt et al. Conversion of acetaminophen to the bioactive N-acylphenolamine AM404 via fatty acid amide hydrolase-dependent arachidonic acid conjugation in the nervous system. J Biol Chem. 2005 Sep 9;280(36):31405-12. doi: 10.1074/jbc.M501489200. Epub 2005 Jun 29. PMID: 15987694.

[74] De Petrocellis et al. Overlap between the ligand recognition properties of the anandamide transporter and the VR1 vanilloid receptor: inhibitors of anandamide uptake with negligible capsaicin-like activity. FEBS Lett. 2000 Oct 13;483(1):52-6. doi: 10.1016/s0014-5793(00)02082-2. PMID: 11033355.

[75] Marsicano et al. The endogenous cannabinoid system controls extinction of aversive memories. Nature. 2002 Aug 1;418(6897):530-4. doi: 10.1038/nature00839. PMID: 12152079.

without anything bad happening, so they eventually learn to dissociate the tone or light from a bad experience, resulting in extinction of the aversive memory. Marsicano et al. tested CB1 mutant (-/-) and wildtype (+/+) by exposing them to a sound, then getting a small electric shock to their feet.[75] After the mice learned this association, they froze or stopped moving when they heard the sound, a sign of fear or heightened awareness. The researchers found both groups of mice learned the association between the sound and the shock equally well. However, when the researchers played the sound without giving the electric shock, the wildtype mice gradually stopped freezing, while the CB1(-/-) mice continued to freeze. These data indicate that CB1 and cannabinoid signaling is important for the process of forgetting/extinguishing fearful/aversive memories.

The impact of cannabinoids has also been shown to have regional or cell type specific effects. Using conditional mutant mice allows genetic mutations to be produced or coupled to the expression of a specific gene which may only be expressed in a specific region of the brain or body. In a study by Lafenêtre et al., mutant mice were produced that lacked CB1 in the cortical glutamatergic neurons or GABAergic neurons.[76] Testing utilized novel palatable food or a novel object and the responses to these stimuli were analyzed in mutant and wildtype mice over several days. The results of this study indicate that cannabinoid signaling can exert region-specific functions and balances between novelty seeking and behavioral inhibition. Specifically, loss of CB1 receptors expressed in cortical glutamatergic neurons produced a delay or latency to approach and eat novel food, and loss of CB1 receptor in inhibitory GABAergic neurons resulted in increased palatable food consumption, similar interactions with novel objects, and increasing novelty seeking behaviors. The authors described the importance of this work in relation to ADHD,

> The balance between novelty seeking and safety assessment is a key feature of adaptive behavior, and alterations in this equilibrium can lead to neuropsychiatric disorders. Excessive novelty seeking is a main form of pathological impulsivity, which is among the symptoms that define attention deficit hyperactivity disorder (ADHD).

Based on these studies, a variety of learning and adaptive behaviors can also be influenced by cannabinoid signaling. As indicated below, APAP can also influence similar testing and behavioral outcomes in animal models with pre, peri, or postnatal exposures. Regarding molecular interactions with APAP and its metabolites, the influence is diverse and includes COX inhibition, and the serotonergic, vanilloid, and cannabinoid signaling pathways are also part of the pharmacological mechanisms of action.[77]

Acetaminophen Toxicity

The Merck Manual reports the following summary for oral route APAP exposure,

> Immediate-release acetaminophen is rapidly and almost completely absorbed from the gastrointestinal (GI) tract, primarily the small intestine. Bioavailability ranges from 85% to 98%. Peak plasma concentrations occur within 30 to 60 minutes and range from 7.7 to 17.6 mcg/mL

[76] Lafenêtre et al. Bidirectional regulation of novelty-induced behavioral inhibition by the endocannabinoid system. Neuropharmacology. 2009 Dec;57(7-8):715-21. doi: 10.1016/j.neuropharm.2009.07.014. Epub 2009 Jul 14. PMID: 19607846. and

[77] Ohashi and Kohno. Analgesic Effect of Acetaminophen: A Review of Known and Novel Mechanisms of Action. Front Pharmacol. 2020 Nov 30;11:580289. doi: 10.3389/fphar.2020.580289. PMID: 33328986; PMCID: PMC7734311.

> after a single 1,000 mg dose and 7.9 to 27 mcg/mL at steady state after 1,000 mg every 6 hours in adult patients….

This estimate is based on a single therapeutic dose (1000mg), which is a human reference dose of 16.7mg/Kg, based on a 60Kg reference human, and

> To cause toxicity, an acute oral overdose must total ≥ 150 mg/kg (about 7.5 g in adults) within 24 hours.[78]

The Merck Manual reports an acute toxic dose is 7.5 grams over 24hrs, which is less than twice the maximum recommended dose of 4 grams (reference human dose of 66.6mg/Kg/day) over 24 hours. It is also noted that aspartate aminotransferase (AST) and alanine aminotransferase (ALT), being liver enzymes, are reported to correlate with the stage of poisoning,

> "Low-level transaminase elevations (e.g., up to 2 or 3 times the upper limit of normal) may occur in adults taking therapeutic doses of acetaminophen for days or weeks. These elevations appear to be transient, usually resolve or decrease within a few days (even with continued acetaminophen use), are usually clinically asymptomatic, and are probably insignificant."

Regarding liver enzymes,

> "If AST and ALT levels are normal (< 50 IU/L [0.83 microkat/L]), and the acetaminophen level is < 10 mcg/mL (< 66 micromol/L), significant hepatotoxicity is very unlikely.
>
> If AST and ALT levels are normal but the acetaminophen level is ≥ 10 mcg/mL (> 66 micromol/L), significant hepatotoxicity is possible; AST and ALT levels are remeasured after 24 hours. If repeat AST and ALT levels are normal, significant hepatotoxicity is unlikely; if the levels are high, significant hepatotoxicity is assumed.
>
> If initial AST and ALT levels are high, regardless of the acetaminophen level, significant hepatotoxicity is assumed."

These reference summaries on therapeutic and toxic dosages, metabolism, and resulting blood concentrations are important to consider regarding dosages with reported human toxicity in the literature and dosages used in animal model toxicity studies, reviewed below.

In regard to AST and ALT concentrations with APAP overdose in humans, initial clinical reports indicated there were elevations in liver enzymes, increased serum insulin, and altered glucose and lactate metabolism.[79,80] Overdosing estimates ranged from 12-50 grams, and the resulting AST ranged from 236 to >5000 IU/L. By the early 1970s, reported hepatotoxicity was recognized as the major manifestation of

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[78] Acetaminophen Poisoning (N-acetyl-para-aminophenol; APAP). By Gerald F. O'Malley , DO, Grand Strand Regional Medical Center; Rika O'Malley , MD, Grand Strand Medical Center. Last review/revision Jun 2022 | Modified Sep 2022

[79] Record et al. Disturbances in glucose metabolism in patients with liver damage due to paracetamol overdose. Clin Sci Mol Med. 1975 Nov;49(5):473-9. doi: 10.1042/cs0490473. PMID: 1192705.

[80] Record et al. Disturbances of lactate metabolism in patients with liver damage due to paracetamol overdose. Metabolism. 1981 Jul;30(7):638-43. doi: 10.1016/0026-0495(81)90076-7. PMID: 7242370.

APAP overdose in experimental animals and humans. The treatment for APAP overdose or poisoning is currently N-acetylcysteine (NAC). This remedy was initially reported and established in mice. Briefly, a lethal dose caused death in ~80% of mice (1200mg/Kg, ~LD80), but treatment with NAC at an equal mass dose (1200mg/Kg) within a few hours of APAP exposure (<4.5hrs) resulted in 85-100% rescue.[81] The following year, 1976, a clinical study reported the utility of NAC in preventing liver damage caused by APAP overdose if treatment was started within 10hrs of APAP exposure.[82] In patients starting treatment after 10hrs, liver damage was reported and indicated by AST or ALT above 1,000 IU/L, with all of these patients reaching 10,000 IU/L for AST or ALT.

Human Equivalent Dosages

To compare the effects of chemical exposures between humans and animal models, dosages are adjusted based on established metabolic differences. Metabolism is generally faster in smaller animals, so animal dosages (mg/Kg) require adjustment to produce comparable human dosages (mg/Kg). For example, a study can administer 50mg of a drug per kilogram of bodyweight (50mg/Kg). While this dosage could be used on humans or animals directly, adjustments for bodyweight (in Kg) are not sufficient to account for differences in metabolism. As indicated above, toxicity risk increases with APAP human dosages at 150 mg/kg, while it takes ~950-1200 mg/kg (PO) to produce a lethal dose to 50% (LD_{50}) of mice.[83] **Animal-human dosages (mg/Kg) should not be directly compared, because differences in size and metabolism necessitate a conversion.** These conversions produce Human Equivalent Dosages (HED) from animal dosages. The U.S. Department of Health and Human Services Food and Drug Administration (FDA) Center for Drug Evaluation and Research (CDER) has provided Guidelines for Industry covering this topic, see Estimating the Maximum Safe Starting Dose in Initial Clinical Trials for Therapeutics in Adult Healthy Volunteers (Table 1).[84] As indicated below, a dose in mice (mg/Kg) can be converted to a HED by dividing it by 12.3, and a mg/Kg dose in rats can be converted to a HED by dividing by 6.2.

[81] Piperno and Berssenbruegge. Reversal of experimental paracetamol toxicosis with N-acetylcysteine. Lancet. 1976 Oct 2;2(7988):738-9. doi: 10.1016/s0140-6736(76)90030-1. PMID: 61415.
[82] Prescott et al. Treatment of paracetamol (acetaminophen) poisoning with N-acetylcysteine. Lancet. 1977 Aug 27;2(8035):432-4. doi: 10.1016/s0140-6736(77)90612-2. PMID: 70646.
[83] Guasch et al. Pharmaco-toxicological effects of acetaminophen in rodents. Battery of tests to screen potential analgesic acetaminophen derivatives. Methods Find Exp Clin Pharmacol. 1990 Mar;12(2):141-8. PMID: 2319838.
[84] Estimating the Maximum Safe Starting Dose in Initial Clinical Trials for Therapeutics in Adult Healthy Volunteers, JULY 2005: https://www.fda.gov/media/72309/download

Table 1

Table 1: Conversion of Animal Doses to Human Equivalent Doses Based on Body Surface Area

Species	To Convert Animal Dose in mg/kg to Dose in mg/m², Multiply by k_m	To Convert Animal Dose in mg/kg to HED[a] in mg/kg, Either:	
		Divide Animal Dose By	Multiply Animal Dose By
Human	37	---	---
Child (20 kg)[b]	25	---	---
Mouse	3	12.3	0.08
Hamster	5	7.4	0.13
Rat	6	6.2	0.16
Ferret	7	5.3	0.19
Guinea pig	8	4.6	0.22
Rabbit	12	3.1	0.32
Dog	20	1.8	0.54
Primates:			
Monkeys[c]	12	3.1	0.32
Marmoset	6	6.2	0.16
Squirrel monkey	7	5.3	0.19
Baboon	20	1.8	0.54
Micro-pig	27	1.4	0.73
Mini-pig	35	1.1	0.95

[a] Assumes 60 kg human. For species not listed or for weights outside the standard ranges, HED can be calculated from the following formula:

HED = animal dose in mg/kg x (animal weight in kg/human weight in kg)$^{0.33}$.

[b] This k_m value is provided for reference only since healthy children will rarely be volunteers for phase 1 trials.

[c] For example, cynomolgus, rhesus, and stumptail.

The HED conversion table above also includes factors for converting between adult humans and children. In addition to differences in surface area, there are often related differences in metabolism and metabolic enzymes between the same species at different developmental stages: embryos, fetuses, newborns, juveniles, and adults. For example, it was recognized in the 1970s, that a cytochrome P-450 enzyme produced a chemically reactive intermediate from APAP, but it would take several years for NAPQI to be postulated and identified.[85] These scientists also reasoned that because the livers in young animals are immature, and expressed fewer cytochrome P-450 enzymes, young animals were expected to be relatively resistant or less vulnerable to APAP hepatotoxicity. This hypothesis was tested and supported in both mice and rats (Table 2).

[85] Reviewed in Hinson. Reactive metabolites of phenacetin and acetaminophen: a review. Environ Health Perspect. 1983 Mar;49:71-9. doi: 10.1289/ehp.834971. PMID: 6339229; PMCID: PMC1569121.

Age	24 Hour LD_{50} Mouse	Rat
7 days	3850(3500-4235)	2350(1942-2840)
14 days	2100(1590-2772)	1200(930-1606)
21 days	2150(1653-2795)	1700(1214-2180)
28 days	1500(1000-1960)	1500(1230-1900)
Adults	800(320-1760)	1580(1285-1900)

LD_{50} in mg/kg 24 hours after ip administration

( )=95% confidence limits

Table 2. Acetaminophen (APAP) Induced Lethality in Mice and Rats by Age. Higher dosages of APAP were required to produce a lethal dose (LD50) in younger mice or rats.[86]

Based on these LD50 data from adult mice and rats, the HED is estimated at 65mg/Kg (800mg/12.3) and 255 mg/Kg (1580mg/6.2), and 313mg/Kg and 379mg/Kg from 7-day old animals, respectively. In all cases, these calculations put the calculated HEDs (65-379mg/Kg) within the range of potential toxicity in humans (≥ 150 mg/kg).

The HED concept is important because dosages from animal studies should be converted based on body surface area and metabolism prior to comparing with human dosages, and these converted results allow researchers to appropriately compare results between humans and the various animals used in experimental studies.

As dosing humans with hepatoxic dosages is unethical, animal studies have been used to examine hepatic necrosis caused by various APAP dosages. For example, a dose of 400 or 600mg/Kg IP in Long Evans male rats resulted in liver necrosis in a minority (20-45%) of animals with no deaths reported, but at 800mg/Kg a majority developed necrosis and 10% died. This LD10 dosage produces a calculated HED of 129mg/Kg (800mg/6.2). At 1000mg/Kg (HED 161mg/Kg), all animals showed variable amounts of liver necrosis and mortality was 33%.[87] This range of toxicity in rats from 800mg-1000mg/Kg (LD10-LD30) produces HEDs from 129-161mg/Kg, and this encompasses the referenced potential for a human toxic dose reported in the Merck Manual, ≥ 150 mg/kg (about 7.5 g in adults). For clarification, a 7.5-gram overdose for a reference human of 60Kg (132lbs) produces a dose of 125mg/Kg. Using these estimates, the reported rat toxicities can be used for rat-human dosage conversion and produce overlapping HEDs and estimated toxic human dosages for APAP.

[86] Mancini et al. Developmental susceptibility to acetaminophen toxicity. Res Commun Chem Pathol Pharmacol. 1980 Mar;27(3):603-6. PMID: 7384649.

[87] Price and Jollow . Increased resistance of diabetic rats to acetaminophen-induced hepatotoxicity. J Pharmacol Exp Ther. 1982 Mar;220(3):504-13. PMID: 7062262.

Dosing humans therapeutically, based on packaging information, is 1 gram in a reference human adult (60Kg), an exposure of 16.7mg/Kg. Calculated therapeutic doses based on HEDs are 16.7mg/Kg X 12.3 = 205 mg/Kg (mouse) and 16.7mg/Kg X 6.2 = 104 mg/Kg (rat). Actual therapeutic doses have been determined experimentally in animals for various antinociceptive, pain relief, effects. For example, a therapeutic mouse median effective dose (ED_{50}) is 185.52 mg/Kg (electrical tail stimulation in mice), and a therapeutic rat ED_{50} is 154.8 mg/Kg (Randall-Selitto's test in rats).[83] In this example, the electrical tail stimulation test in mice is within 10% of the calculated mouse HED and the Randall-Selitto's test (paw pressure test) is within 50% of the rat HED. Similarly, the mouse ED_{50} of 186mg/Kg divided by 12.3 = 15.1mg/Kg (HED) and the rat ED_{50} of 155mg/Kg divided by 6.2 = 25mg/Kg (HED), and the package information is 16.7mg/Kg (actual human dose). Remarkably, a human dose is between the experimentally determined median effective doses from these animal models converted to HEDs 15.1-25mg/kg. **Based on these data and calculations, a mouse dose of ~150-200mg/Kg or a rat dose of ~100-150 mg/Kg is therapeutic, as reported from experimental studies and calculated using HED conversions.**

In summary, one should not compare mg/Kg dosing directly between different species. The HED conversion should be used to account for biological and metabolic differences between different species when comparing human and animal studies. These conversions are consistent with industry guidelines and allow reasonable estimates to be calculated from human dosages, for equivalent dosing in research animals, and they can also be used in the reverse, to convert dosages in animal research to human equivalent doses (HED).

THE DEVELOPMENTAL AND REPRODUCTIVE TOXICITY OF ACETAMINOPHEN

Adverse Outcome Pathway for Oxidative Stress and Developmental Impairment in Learning in Memory

The Organization for Economic Co-operation and Development (OECD) is an international organization, in which the U.S. participates through the Environmental Protection Agency, that analyzes causal relationships between chemical exposures and adverse outcomes in humans. The OECD prepared an Adverse Outcome Pathway analysis on a set of chemicals that bind to certain proteins, in particular to glutathione.[88] The results were published December 15, 2022, as AOP 20. This analysis is relevant here because one of the identified stressor chemicals for AOP 20 is acetaminophen, and the analysis concluded that exposure to these chemicals during "brain development" may create "oxidative stress" sufficient to "cause cellular injury and death" that disrupts "the establishment of neuronal connections and networks" and can "lead to functional impairment in learning and memory." AOP 20 Abstract.

This AOP report is titled, "Binding of electrophilic chemicals to SH (thiol)-group of proteins and /or to seleno-proteins involved in protection against oxidative stress during brain development leading to impairment of learning and memory."[89] It is AOP 20 in the OECD Series on Adverse Outcome Pathways (Figure 11). This AOP is endorsed by regulatory authorities, including the National Coordinators of the Test Guidelines Programme (WNT) and the Working Party on Hazard Assessment (WPHA). The United States and numerous other countries are member countries for the OECD.[90]

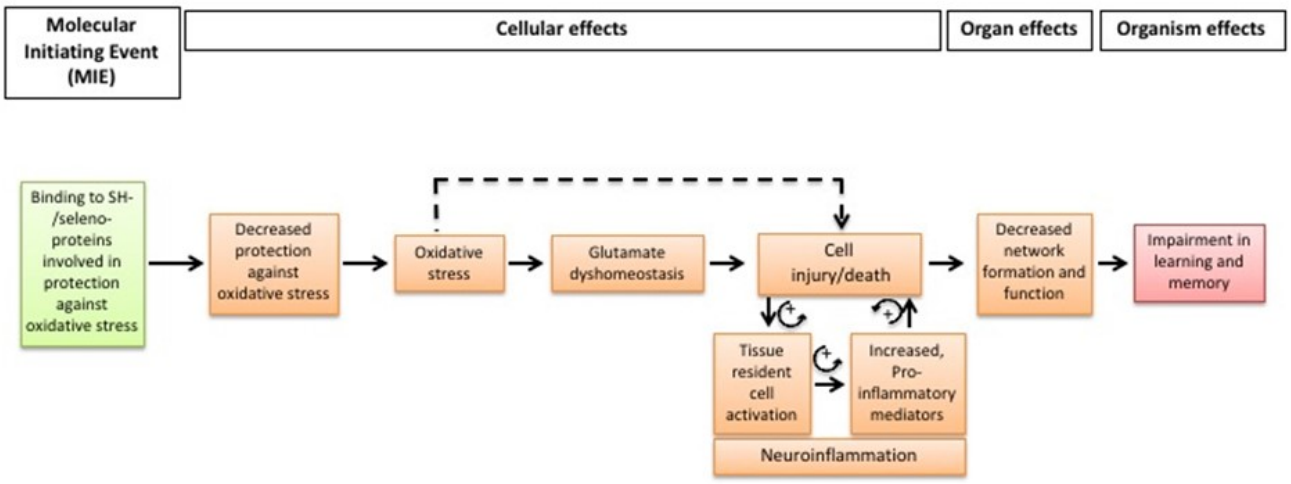

Figure 11. Oxidative Stress and Developmental Impairment of Learning and Memory

The abstract for the OECD AOP 20 reads as follows,

[88] Tschudi-Monnet et al. Binding of electrophilic chemicals to SH(thiol)-group of proteins and /or to seleno-proteins involved in protection against oxidative stress during brain development leading to impairment of learning and memory, OECD Series on Adverse Outcome Pathways, No. 20, OECD Publishing, Paris, 2022. https://doi.org/10.1787/4df0e9e4-en.
[89] Id.
[90] *National co-ordinators of the Test Guidelines programme*, ORG. FOR ECON. CO-OPERATION AND DEV., https://www.oecd.org/chemicalsafety/testing/national-coordinators-test-guidelines-programme.htm.

> This Adverse Outcome Pathway (AOP) describes the linkage between binding to sulfhydryl (SH)-/seleno-proteins involved in protection against oxidative stress and impairment in learning and memory, the Adverse Outcome (AO). Binding to SH-/ seleno-proteins involved in protection against oxidative stress has been defined as the Molecular Initiating Event (MIE). Production, binding and degradation of Reactive Oxygen Radicals (ROS) are tightly regulated, and an imbalance between production and protection may cause oxidative stress, which is common to many toxicity pathways. Oxidative stress may lead to an imbalance in glutamate neurotransmission, which is involved in learning and memory. **Oxidative stress may also cause cellular injury and death. During brain development and in particular during the establishment of neuronal connections and networks, such perturbations may lead to functional impairment in learning and memory.** Neuroinflammation (Resident cell activation; Increased pro-inflammatory mediators) is triggered early in cell injury cascades and is considered as an exacerbating factor. The weight-of-evidence supporting the relationship between the described key events is based mainly on developmental effects observed after an exposure to the heavy metal, mercury, known for its strong affinity to many SH-/seleno-containing proteins, but in particular to those having anti-oxidant properties, such as glutathione (GSH). The overall assessment of this AOP is considered as strong, based on the biological plausibility, the empirical support and on the essentiality of the Key Events (KEs), which are moderate to strong, since blocking, preventing or attenuating an upstream KE is mitigating the downstream KE. The gap of knowledge is mainly due to limited quantitative evaluations, impeding thus the development of predictive models.

AOP 20 identifies acetaminophen as a stressor chemical for which the adverse outcome pathway applies (p 42, Event 1392: Oxidative Stress). The report also indicates that APAP produces oxidative stress as a key event. This is not unexpected, as APAP is known to produce hepatotoxicity via NAPQI and oxidative damage, which can be mitigated by NAC, as indicated above in **Acetaminophen Toxicity**. The OECD AOP 20 report is "based mainly on developmental effects observed after an exposure to the heavy metal, mercury…". The listed prototypical stressors for this AOP are methylmercuric (II) chloride, mercuric chloride, and acrylamide. The AOP describes oxidative stress: (p 43).

> Oxidative stress is defined as an imbalance in the production of reactive oxygen species (ROS) and antioxidant defenses. High levels of oxidizing free radicals can be very damaging to cells and molecules within the cell...

Regarding the potential impacts of this oxidative stress, the AOP continues,

> …The brain possesses several key physiological features, such as high O_2 utilization, high polyunsaturated fatty acids content, presence of autooxidable neurotransmitters, and low antioxidant defenses as compared to other organs, that make it highly susceptible to oxidative stress…

The OECD AOP 20 provides a weight-of-evidence analysis for the stressor mercury. By incorporating APAP as a stressor, the AOP provides independent support for a causal relationship between APAP and the identified key events and adverse outcomes. Oxidative stress, cell injury, and decreased neuronal network function are included as key events, and the common adverse outcomes are impairment in learning and memory. These adverse effects are related to, but not specific for, neurodevelopmental

disorder. Specifically, the Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition (DSM-5) defines neurodevelopmental disorders as Intellectual Developmental Disorder (IDD), communication disorders, autism spectrum disorder (ASD), attention deficit hyperactivity disorder (ADHD), specific learning disorder, and motor disorders.[91]

Regarding the definition and regulatory significance of impairment in learning and memory as an AO (p 97), the AOP also provides guidance,

> A prime example of impairments in learning and memory as the adverse outcome for regulatory action is developmental lead exposure and IQ function in children (Bellinger, 2012). Most methods are well established in the published literature and many have been engaged to evaluate the effects of developmental thyroid disruption. The US EPA and OECD Developmental Neurotoxicity (DNT) Guidelines (OCSPP 870.6300 or OECD TG 426) as well as OECD TG 443 (OECD, 2018) require testing of learning and memory (USEPA, 1998; OECD, 2007) advising to use the following tests passive avoidance, delayed-matching-to-position for the adult rat and for the infant rat, olfactory conditioning, Morris water maze, Biel or Cincinnati maze, radial arm maze, T-maze, and acquisition and retention of schedule-controlled behaviour. These DNT Guidelines have been deemed valid to identify developmental neurotoxicity and adverse neurodevelopmental outcomes (Makris et al., 2009). Also in the frame of the OECD GD 43 (2008) on reproductive toxicity, learning and memory testing may have potential to be applied in the context of developmental neurotoxicity studies.

This AOP clearly indicates oxidative stress as a KE, that oxidative stress can produce cell injury, cell injury can result in decreased neuronal network function, and thereby cause impairment in learning and memory. The AOP also defines examples of impairments in learning and memory in children and animal models. Regarding the ability of oxidative stress to cause neurodevelopmental disorders, the AOP indicates (p 185) oxidative stress,

> …has also been linked to neurodevelopmental diseases and deficits like autism spectrum disorder and postnatal motor coordination deficits...

Based on this information, there is compelling evidence and support for APAP to cause impairment in learning and memory, but additionally, APAP specific analysis is warranted. This report will therefore apply this AOP framework to APAP, **focusing on the developmental effects observed after an exposure to APAP,** and determine the weight-of-evidence for a causal relationship between APAP and the neurodevelopmental disorders ASD and ADHD.

DNA Oxidation in Reproductive, Developmental, and Neurodevelopmental Toxicity.

APAP and metabolite NAPQI are sufficient to cause oxidative stress and damage in a dose-dependent manner. This oxidative damage affects the normal functioning of cells, leading to changes in cell behavior. When the level of oxidative damage becomes too high, it can result in cell death/apoptosis. The brain is particularly vulnerable to oxidative stress, and during pregnancy, the human brain produces NAPQI due

[91] American Psychiatric Association. (2013). Diagnostic and statistical manual of mental disorders (5th ed.). Washington, DC: Author.

to the presence of CYP2E1.[92, 93] Oxidative damage can also impact the development of the nervous system, leading to potential negative effects on learning, memory, and behavior, including neurodevelopmental disorders like ASD and ADHD. Additionally, oxidative damage can affect other organs, including reproductive tissues in males, specifically epididymal cells.[94] Since the central nervous system and reproductive organs develop throughout pregnancy, exposures to APAP that result in oxidative DNA damage can potentially cause reproductive, developmental, and neurodevelopmental toxicity in cells and tissues where APAP and NAPQI have interactions. These outcomes overlap in preclinical testing, as there are interconnected relationships between oxidative DNA damage, reproductive impacts, development, neurodevelopment, reproductive behaviors, and the potential for multi-generational effects and effects on offspring exposed *in utero*.

AOP: Acetaminophen Causes Oxidative Stress and Neurodevelopmental Disorders

This Adverse Outcome Pathway (AOP) describes the linkage between acetaminophen, metabolites of acetaminophen, glutathione, and neurodevelopmental disorders. Acetaminophen is known to undergo metabolism into two metabolites that result in downstream interactions: NAPQI and AM404. NAPQI, a benzoquinone radical, is a toxic metabolite of APAP that reacts with thiols and sulfhydryl (-SH) groups. AM404 is a metabolite of APAP that alters cannabinoid and serotonergic signaling. Both cannabinoid and serotonergic signaling are essential for normal autonomic and neuronal development. In the following sections, I examine the impact of APAP on serotonergic signaling, AM404 on cannabinoid signaling, and NAPQI on oxidative stress for causal physical and chemical interactions that produce impairment in learning, social behavior, focusing, repetitive behavior, and hyperactivity and neurodevelopmental disorders, in particular ASD and ADHD, the Adverse Outcomes (AOs). The resulting AOP outlines a causal pathway initiated by (1) molecular interactions, on to (2) cellular events, to (3) tissue and organ events, to (4) effects on the organism phenotype and behavior.

1. Molecular Interactions

A. Metabolism of Acetaminophen

APAP is metabolized by three major routes and one more recently discovered minor pathway (see **Acetaminophen Metabolism**, above). These metabolic pathways and associated enzymes (proteins) are glucuronidation (UGT1A1, UGT1A6)[95], sulfation (SULT1A1, SULT1A3, SULT1E1)[96], oxidation (CYP2E1)[97], and deacetylation-hydrolase-arachidonic acid conjugation, by arylacetamide deacetylase

[92] Brzezinski et al. Catalytic activity and quantitation of cytochrome P-450 2E1 in prenatal human brain. J Pharmacol Exp Ther. 1999 Jun;289(3):1648-53. PMID: 10336564.
[93] Boutelet-Bochan et al. Expression of CYP2E1 during embryogenesis and fetogenesis in human cephalic tissues: implications for the fetal alcohol syndrome. Biochem Biophys Res Commun. 1997 Sep 18;238(2):443-7. doi: 10.1006/bbrc.1997.7296. PMID: 9299528.
[94] Katen et al. Epididymal CYP2E1 plays a critical role in acrylamide-induced DNA damage in spermatozoa and paternally mediated embryonic resorptions. Biol Reprod. 2017 Apr 1;96(4):921-935. doi: 10.1093/biolre/iox021. PMID: 28379345.
[95] Reviewed in Bock and Köhle. UDP-glucuronosyltransferase 1A6: structural, functional, and regulatory aspects. Methods Enzymol. 2005;400:57-75. doi: 10.1016/S0076-6879(05)00004-2. PMID: 16399343.
[96] Adjei et al. Interindividual variability in acetaminophen sulfation by human fetal liver: implications for pharmacogenetic investigations of drug-induced birth defects. Birth Defects Res A Clin Mol Teratol. 2008 Mar;82(3):155-65. doi: 10.1002/bdra.20535. PMID: 18232020.
[97] Reviewed in Tanaka E. In vivo age-related changes in hepatic drug-oxidizing capacity in humans. J Clin Pharm Ther. 1998 Aug;23(4):247-55. doi: 10.1046/j.1365-2710.1998.00164.x. PMID: 9867310.

(AADAC) and fatty acid amide hydrolase (FAAH).[98] The glucuronidation, sulfation, and oxidation pathways have been known since the 1970s,[99] with the role of AADAC on APAP deacetylation-hydrolysis being characterized most recently in 2010,[100] following the identification of arachidonic acid conjugation of PAP by FAAH in 2005.[101]

The relative composition of APAP metabolites results in production of 50-70% APAP-glucuronide, 25-35% APAP-sulfate, 5-15% NAPQI,[102] and 1-2% PAP,[103] 0.001-0.009% AM404,[104] see **Acetaminophen Metabolism**, above. While glucuronide and sulfate maintain the bulk of APAP metabolism and vary within populations, the reported toxic and therapeutic mechanisms of action for APAP are based on the interactions of NAPQI and AM404.

The production of NAPQI from APAP has been a known metabolite and risk factor for oxidative damage and liver necrosis since the 1970-80s.[105] The oxidation of APAP by the cytochrome P450 enzyme (CYP2E1) found in the liver results in the production of NAPQI. Initial reports indicated that CYP2E1, 1A2, and 3A4 were all implicated in the formation of NAPQI.[106] Additional pharmacokinetic and pharmacogenetic studies indicated that CYP2E1 is the primary enzyme involved in APAP oxidation and 1A2 and 3A4 have negligible impacts *in vivo* on APAP metabolism into NAPQI.[107]

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[98] Högestätt et al. Conversion of acetaminophen to the bioactive N-acylphenolamine AM404 via fatty acid amide hydrolase-dependent arachidonic acid conjugation in the nervous system. J Biol Chem. 2005 Sep 9;280(36):31405-12. doi: 10.1074/jbc.M501489200. Epub 2005 Jun 29. PMID: 15987694.

[99] Levy and Regårdh. Drug biotransformation interactions in man. V. Acetaminophen and salicylic acid. J Pharm Sci. 1971 Apr;60(4):608-11. doi: 10.1002/jps.2600600423. PMID: 4942784.; Mitchell et al. Acetaminophen-induced hepatic necrosis. I. Role of drug metabolism. J Pharmacol Exp Ther. 1973 Oct;187(1):185-94. PMID: 4746326.

[100] Watanabe et al. Arylacetamide deacetylase is a determinant enzyme for the difference in hydrolase activities of phenacetin and acetaminophen. Drug Metab Dispos. 2010 Sep;38(9):1532-7. doi: 10.1124/dmd.110.033720. Epub 2010 Jun 11. PMID: 20542992.

[101] Högestätt et al. Conversion of acetaminophen to the bioactive N-acylphenolamine AM404 via fatty acid amide hydrolase-dependent arachidonic acid conjugation in the nervous system. J Biol Chem. 2005 Sep 9;280(36):31405-12. doi: 10.1074/jbc.M501489200. Epub 2005 Jun 29. PMID: 15987694.

[102] Reviewed in Ghanem et al. Acetaminophen from liver to brain: New insights into drug pharmacological action and toxicity. Pharmacol Res. 2016 Jul;109:119-31. doi: 10.1016/j.phrs.2016.02.020. Epub 2016 Feb 26. PMID: 26921661; PMCID: PMC4912877.

[103] Nicholls et al. NMR and HPLC-NMR spectroscopic studies of futile deacetylation in paracetamol metabolites in rat and man. J Pharm Biomed Anal. 1997 Apr;15(7):901-10. doi: 10.1016/s0731-7085(96)01950-4. PMID: 9160256.; Sharma et al. First evidence of the conversion of paracetamol to AM404 in human cerebrospinal fluid. J Pain Res. 2017 Nov 28;10:2703-2709. doi: 10.2147/JPR.S143500. PMID: 29238213; PMCID: PMC5716395.

[104] Muramatsu et al. Metabolism of AM404 From Acetaminophen at Human Therapeutic Dosages in the Rat Brain. Anesth Pain Med. 2016 Jan 17;6(1):e32873. doi: 10.5812/aapm.32873. PMID: 27110534; PMCID: PMC4834746.

[105] Reviewed in Hinson. Reactive metabolites of phenacetin and acetaminophen: a review. Environ Health Perspect. 1983 Mar;49:71-9. doi: 10.1289/ehp.834971. PMID: 6339229; PMCID: PMC1569121.

[106] Lee et al. Role of CYP2E1 in the hepatotoxicity of acetaminophen. J Biol Chem. 1996 May 17;271(20):12063-7. doi: 10.1074/jbc.271.20.12063. PMID: 8662637.; Patten et al. Cytochrome P450 enzymes involved in acetaminophen activation by rat and human liver microsomes and their kinetics. Chem Res Toxicol. 1993 Jul-Aug;6(4):511-8. doi: 10.1021/tx00034a019. PMID: 8374050.

[107] Manyike et al. Contribution of CYP2E1 and CYP3A to acetaminophen reactive metabolite formation. Clin Pharmacol Ther. 2000 Mar;67(3):275-82. doi: 10.1067/mcp.2000.104736. PMID: 10741631.; Zaher et al. Protection against acetaminophen toxicity in CYP1A2 and CYP2E1 double-null mice. Toxicol Appl Pharmacol. 1998 Sep;152(1):193-9. doi: 10.1006/taap.1998.8501. PMID: 9772215.

B. Downstream Interactions of NAPQI

NAPQI, the primary toxic metabolite of APAP, is a "radical." A radical (also known as reactive oxygen species or ROS) is a molecule with at least one unpaired electron that is highly reactive and can bind with other molecules. When a radical binds with a molecule, the molecule is said to be "oxidized." A common example of oxidation is rust that appears on metals. Antioxidants are molecules that can bind with free radicals and "scavenge" the radicals before they cause damage by oxidation. Oxidative stress occurs in cells when insufficient antioxidant molecules are available to counteract the effect of unstable free radical molecules.

Glutathione is an antioxidant created in the body that serves as a primary cellular defense against free radicals. Glutathione can mitigate the damage caused by NAPQI. A molecule of glutathione that has not been oxidized by a radical is called "reduced" glutathione. Reduced glutathione (GSH) is made from a tripeptide of glutamine, cysteine, and glycine. The GSH molecule can be oxidized at the -SH group on the cysteinyl amino acid, resulting in oxidized glutathione (GSSG), which accounts for its antioxidant properties and interaction with NAPQI. GSH is particularly important for the brain, because brain tissue is highly susceptible to oxidative stress due to its high content of unsaturated fatty acids, high oxygen consumption, and poorly developed oxidative defense mechanisms.

A single 1,000 milligram dose of APAP can produce 50-150 mg (5-15%) of toxic NAPQI radicals. Free radical and reactive oxygen species (ROS) produce an oxidative environment inside a cell and can thereby deplete GSH. When GSH is depleted, free radicals—including NAPQI and other endogenous radicals that normally rely on GSH buffering—are unimpeded and can cause damage that disrupts normal metabolic, cellular, and neurodevelopment of brain tissues in multiple different ways. (In later sections, I describe how NAPQI binds to GSH, proteins, and enzymes and disrupts normal neural development.)

Because ROS are unstable, their presence in cells is difficult to measure directly. In the published AOP (WPHA/WNT Endorsed), the presence of oxidative stress was examined through indirect measurements of ROS damage. These include measuring glutathione (GSH), the ratio of reduced to oxidized GSH (GSH:GSSG), lipid peroxidation using thiobarbituric acid reactive substances (TBARS), and oxidative DNA damage (8-oxo-dG adducts). I describe below the impact of APAP in causing oxidative stress based on these same measurements.

i. APAP and GSH

It has been known since the 1970s that APAP results in depletion of GSH with increasing dosages. GSH reacts with APAP, but at increasing dosages APAP reacts with other tissues and proteins, such as liver proteins in mice (Table 3). Similar results have also been reported in the yolk sac of cultured rat embryos exposed to APAP.[108]

[108] Weeks et al. Acetaminophen toxicity to cultured rat embryos. Teratog Carcinog Mutagen. 1990;10(5):361-71. doi: 10.1002/tcm.1770100502. PMID: 1981948.

Dose of Acetaminophen	Hepatic Glutathione[a, b]	Covalent Binding to Liver Protein[a]
mg/kg	*% of initial level*	*nmol bound/mg protein*
10	100 ± 3	0.05 ± 0.04
25	95 ± 4	0.03 ± 0.04
100	76 ± 8	0.04 ± 0.04
200	41 ± 6	0.08 ± 0.05
375	19 ± 2	0.71 ± 0.13
750	17 ± 2	1.89 ± 0.21

Table 3. Inverse Relationship of Acetaminophen (APAP) Dose and Liver Glutathione (GSH). Injection (IP) of APAP from 10-750mg/Kg results in hepatic GSH depletion.[109]

The interactions of APAP are concentration dependent, as demonstrated by the capacity of APAP to be metabolized and the depletion of GSH by APAP as a function of dose (Figure 12). Unmetabolized APAP was observed in the hepatocyte suspensions after 3 h incubation with > 2mM APAP. This was paralleled by hepatocellular GSH decreasing above 0.5 mM APAP and approaching depletion at ≥ 2 mM APAP. As indicated above, TBARS were also formed at this transition (Figure 12, Right).

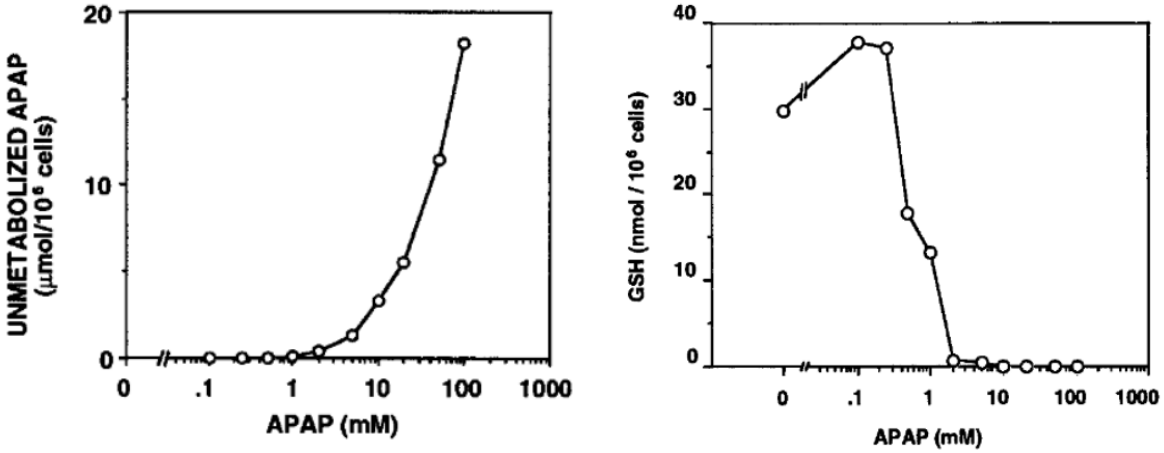

Figure 12. Acetaminophen (APAP) Metabolism and Glutathione (GSH) Interactions. (Left) Unmetabolized APAP in hepatocytes. The unmetabolized APAP concentration in the hepatocyte suspensions incubated for 3 h with APAP was plotted against the initial concentration of APAP. (Right) GSH depletion by APAP in hepatocytes. The hepatocellular GSH concentration was determined in the hepatocytes incubated at 37 °C for 3 h with or without APAP.[111]

ii. APAP and TBARS

Lipid peroxidation is the process that occurs when free radicals attack lipids. A common test for assessing lipid peroxidation is to measure the level of thiobarbituric acid reactive substances (TBARS) formed as a byproduct of lipid peroxidation. APAP at a concentration of 1mM in hepatocytes (liver cells) has been shown to produce a peak concentration of TBARS (Figure 13).[110]

[109] Mitchell et al. Drug metabolism as a cause of drug toxicity. Drug Metab Dispos. 1973 Jan-Feb;1(1):418-23. PMID: 4149413.
[110] By contrast, APAP has been shown to produce a 50% reduction in TBARS at concentration of 1mM in a sarcoplasmic reticulum model. These data indicate that APAP slows down oxidation in the absence of metabolism. See Dinis et al. Action of phenolic derivatives (acetaminophen, salicylate, and 5-aminosalicylate) as inhibitors of membrane lipid peroxidation and as

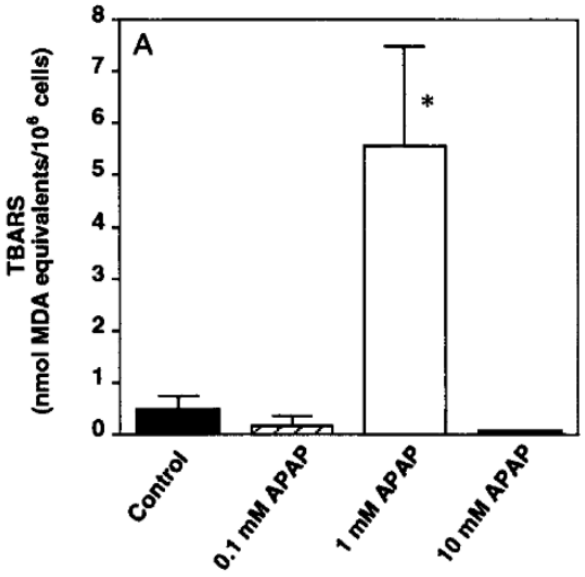

Figure 13. Production of TBARS of Acetaminophen (APAP) is Concentration Dependent. At the low concentration of APAP, 0.1mM, TBARS were not increased. TBARS were produced at 1mM. At the highest concentration, 10mM, unmetabolized APAP was reported to inhibit the formation of TBARS.[111]

In another study, rats exposed to APAP during neurodevelopment at doses equivalent to human therapeutic doses showed significant increased oxidative markers (TBARS) and inflammatory makers.[112] Exposure to APAP (50 mg/kg sc.) on post-natal day 5 (which corresponds to third trimester human development) resulted in the highest increase in TBARS in examined brain tissue.

iii. APAP and DNA Oxidation

Oxidation of DNA can result in oxidized DNA base pairs including 8-hydroxy-deoxyguanosine (8-OH-dG). If not repaired, these oxidized DNA base pairs can cause mutations and/or altered gene expression or epigenetic changes leading to reproductive and developmental toxicity, neurodevelopmental deficits, and cancer.[113] The concentration of oxidized DNA is another measure of oxidative stress. Treatments with APAP at both subtoxic (150mg/kg) and toxic doses (1500mg/Kg and 2000 mg/Kg) were reported to cause a significant increase in DNA oxidation (8-OH-dG) as well as a significant decrease in GSH content in rat liver.**[114]** Increased concentrations of APAP in human cord blood are associated with increased cord

peroxyl radical scavengers. Arch Biochem Biophys. 1994 Nov 15;315(1):161-9. doi: 10.1006/abbi.1994.1485. PMID: 7979394.

[111] Minamide et al. Spontaneous chemiluminescence production, lipid peroxidation, and covalent binding in rat hepatocytes exposed to acetaminophen. J Pharm Sci. 1998 May;87(5):640-6. doi: 10.1021/js9701014. PMID: 9572917.

[112] Saeedan et al. Effect of early natal supplementation of paracetamol on attenuation of exotoxin/endotoxin induced pyrexia and precipitation of autistic like features in albino rats. Inflammopharmacology. 2018 Aug;26(4):951-961. doi: 10.1007/s10787-017-0440-2. Epub 2018 Jan 11. PMID: 29327281.

[113] Wells et al. Oxidative stress in developmental origins of disease: teratogenesis, neurodevelopmental deficits, and cancer. Toxicol Sci. 2009 Mar;108(1):4-18. doi: 10.1093/toxsci/kfn263. Epub 2009 Jan 6. PMID: 19126598.

[114] Powell et al. Phenotypic anchoring of acetaminophen-induced oxidative stress with gene expression profiles in rat liver. Toxicol Sci. 2006 Sep;93(1):213-22. doi: 10.1093/toxsci/kfl030. Epub 2006 Jun 2. PMID: 16751229; PMCID: PMC1805881.

DNA oxidation (8-OH-dG) level.[115] Independent studies have also reported that urinary 8-OH-dG (DNA oxidation) and 8-OH-G (8-hydroxy-guanosine, RNA oxidation) concentrations are positively correlated with APAP exposures.[116] Another study also reported that urinary concentrations of APAP in pregnant women are associated with higher levels of the selected oxidative stress biomarkers: 8-OHG, 8-OHdG, and HNE-MA (4-hydroxy nonenalmercapturic acid).[117]

All of these molecular interactions indicate that APAP or metabolites of APAP, such as NAPQI, cause oxidative stress, DNA damage, and dose responsive GSH depletion, and these data are consistent with the published OECD AOP No. 20 (**Figure 11. Oxidative Stress and Developmental Impairment of Learning and Memory**).[118] As indicated below, oxidative damage is reported to occur at therapeutic dosages, with dose-effects modified by timing, duration, and frequency of exposures.

2. Cellular Interaction

At the cellular level, APAP and its metabolites cause several key events in the adverse outcome pathway that leads to neurodevelopmental damage, ASD and ADHD.

The APAP metabolite NAPQI causes cellular damage.

Free radicals, such as NAPQI, can bind to molecules within cells and damage or disrupt the normal functions of proteins, lipids, DNA, and other components of the cell, resulting in various types of oxidative-damaging impacts. For example, NAPQI can bind to sulfur-containing amino acids, such as cysteines (Cys), that are the building blocks for proteins. Although Cys residues are commonly targeted,[119] it has also been reported that NAPQI may also damage proteins at methionine (Met), tyrosine (Tyr), and tryptophan (Trp) residues.[120] When NAPQI binds to Cys or other amino acids found in proteins, it can alter a structure or function of the protein.

The primary proteins NAPQI is reported to interact with are glutathione S-transferases.[121] Glutathione S-transferases catalyze the reduction or conjugation of GSH to various electrophilic compounds, including NAPQI. NAPQI is reported to interact with a Cys residue in position 49 of the protein, and alkylation of

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[115] Anand et al. Perinatal Acetaminophen Exposure and Childhood Attention-Deficit/Hyperactivity Disorder (ADHD): Exploring the Role of Umbilical Cord Plasma Metabolites in Oxidative Stress Pathways. Brain Sci. 2021 Sep 30;11(10):1302. doi: 10.3390/brainsci11101302. PMID: 34679367; PMCID: PMC8533963.

[116] Sun et al. Urinary concentrations of acetaminophen in young children in central and south China: Repeated measurements and associations with 8-hydroxy-guanosine and 8-hydroxy-2'-deoxyguanosine. Sci Total Environ. 2021 Sep 15;787:147614. doi: 10.1016/j.scitotenv.2021.147614. Epub 2021 May 8. PMID: 33992949.

[117] Li et al. Urinary paracetamol (4-acetaminophenol) and its isomer 2-acetaminophenol of Chinese pregnant women: Exposure characteristics and association with oxidative stress biomarkers. Sci Total Environ. 2022 Dec 15;852:158375. doi: 10.1016/j.scitotenv.2022.158375. Epub 2022 Aug 29. PMID: 36049689.

[118] Tschudi-Monnet et al. Binding of electrophilic chemicals to SH(thiol)-group of proteins and /or to seleno-proteins involved in protection against oxidative stress during brain development leading to impairment of learning and memory, OECD Series on Adverse Outcome Pathways, No. 20, OECD Publishing, Paris, 2022. https://doi.org/10.1787/4df0e9e4-en.

[119] Streeter et al. The covalent binding of acetaminophen to protein. Evidence for cysteine residues as major sites of arylation in vitro. Chem Biol Interact. 1984 Mar;48(3):349-66. doi: 10.1016/0009-2797(84)90145-5. PMID: 6713598.

[120] Leeminget al. What Are the Potential Sites of Protein Arylation by N-Acetyl-p-benzoquinone Imine (NAPQI)? Chem Res Toxicol. 2015 Nov 16;28(11):2224-33. doi: 10.1021/acs.chemrestox.5b00373. Epub 2015 Nov 2. PMID: 26523953.

[121] Coles et al. The spontaneous and enzymatic reaction of N-acetyl-p-benzoquinonimine with glutathione: a stopped-flow kinetic study. Arch Biochem Biophys. 1988 Jul;264(1):253-60. doi: 10.1016/0003-9861(88)90592-9. PMID: 3395122.

this residue is accompanied by marked increase in the specific activity of the enzyme.[122] The predominate form of glutathione S-transferase that conjugates NAPQI is referred to as glutathione S-transferase Pi.[123] Absence of glutathione S-transferase Pi is reported to mitigate APAP toxicity, because its absence limits the ability of NAPQI to deplete GSH.[124] NAPQI has also been reported to bind 10-formyltetrahydrofolate dehydrogenase,[125] also known as aldehyde dehydrogenase 1L1 (ALDH1L1), glutamate dehydrogenase,[126] and aldehyde dehydrogenase.[127] The enzyme ALDH1L1, which is involved in folate metabolism, is found in high amounts in the liver.[128] ALDH1L1 participates in one-carbon metabolism by converting10-formyltetrahydrofolate to tetrahydrofolate. Mice lacking the *Aldh1l1* gene exhibit changes in liver metabolism and show symptoms similar to folate deficiency when they were fed a standard mouse diet.[129]

Both glutamate dehydrogenase and aldehyde dehydrogenase are mitochondrial proteins. NAPQI is taken up by mitochondria, which are the organelles within cells that produce ATP (adenosine triphosphate), the molecule that is used to store and transport cellular-chemical energy.[130] The normal operation of mitochondria produces free radicals that must be scavenged, so mitochondria are particularly vulnerable to ROS and oxidative stress.

NAPQI can also disrupt and damage structural proteins. The interior of cells contains various structural or cytoskeletal proteins, including microfilament proteins (e.g., actin), microtubules (e.g., tubulin), and scaffold proteins (e.g., spectrin). These proteins allow organism movements (myosin-actin), cell movements (microtubules), movement of structures within cells, and scaffolding to shape the cell. NAPQI was reported to decrease spectrin and actin proteins.[131] Spectrin is reported to influence cell adhesion and spreading, formation of lamellipodia, and morphogenetic processes, such as eye development, oogenesis,

[122] Weis et al. N-acetyl-p-benzoquinone imine-induced protein thiol modification in isolated rat hepatocytes. Biochem Pharmacol. 1992 Apr 1;43(7):1493-505. doi: 10.1016/0006-2952(92)90207-y. PMID: 1567474.
[123] Coles et al. The spontaneous and enzymatic reaction of N-acetyl-p-benzoquinonimine with glutathione: a stopped-flow kinetic study. Arch Biochem Biophys. 1988 Jul;264(1):253-60. doi: 10.1016/0003-9861(88)90592-9. PMID: 3395122.
[124] Henderson et al. Increased resistance to acetaminophen hepatotoxicity in mice lacking glutathione S-transferase Pi. Proc Natl Acad Sci U S A. 2000 Nov 7;97(23):12741-5. doi: 10.1073/pnas.220176997. PMID: 11058152; PMCID: PMC18834.
[125] Pumford et al. Covalent binding of acetaminophen to N-10-formyltetrahydrofolate dehydrogenase in mice. J Pharmacol Exp Ther. 1997 Jan;280(1):501-5. PMID: 8996234.
[126] Halmes et al. Glutamate dehydrogenase covalently binds to a reactive metabolite of acetaminophen. Chem Res Toxicol. 1996 Mar;9(2):541-6. doi: 10.1021/tx950158a. PMID: 8839060.
[127] Landin et al Identification of a 54-kDa mitochondrial acetaminophen-binding protein as aldehyde dehydrogenase. Toxicol Appl Pharmacol. 1996 Nov;141(1):299-307. doi: 10.1006/taap.1996.0287. PMID: 8917703.
[128] Krupenko. FDH: an aldehyde dehydrogenase fusion enzyme in folate metabolism. Chem Biol Interact. 2009 Mar 16;178(1-3):84-93. doi: 10.1016/j.cbi.2008.09.007. Epub 2008 Sep 19. PMID: 18848533; PMCID: PMC2664990.
[129] Krupenko NI, Sharma J, Pediaditakis P, Fekry B, Helke KL, Du X, Sumner S, Krupenko SA. Cytosolic 10-formyltetrahydrofolate dehydrogenase regulates glycine metabolism in mouse liver. Sci Rep. 2019 Oct 17;9(1):14937. doi: 10.1038/s41598-019-51397-1. PMID: 31624291; PMCID: PMC6797707; Sharma et al. Sex-Specific Metabolic Effects of Dietary Folate Withdrawal in Wild-Type and Aldh1l1 Knockout Mice. Metabolites. 2022 May 18;12(5):454. doi: 10.3390/metabo12050454. PMID: 35629957; PMCID: PMC9143804.
[130] Ramachandran and Jaeschke. A mitochondrial journey through acetaminophen hepatotoxicity. Food Chem Toxicol. 2020 Jun;140:111282. doi: 10.1016/j.fct.2020.111282. Epub 2020 Mar 21. PMID: 32209353; PMCID: PMC7254872.
[131] Nicotera et al. Differential effects of arylating and oxidizing analogs of N-acetyl-p-benzoquinoneimine on red blood cell membrane proteins. Arch Biochem Biophys. 1990 Nov 15;283(1):200-5. doi: 10.1016/0003-9861(90)90631-8. PMID: 2146923.

and angiogenesis.[132] Actin and bundles of actin referred to as actin cables are essential to neurulation and neural tube closure.[133]

The oxidative stress-induced damage can disrupt normal neural development in multiple ways. As indicated below, it can disrupt migration of cells or the differentiation of stem cells into neurons, resulting in the production of different types of cells, such as glial cells or astrocytes. It can also impede the proliferation of stem cells directly or cause them to differentiate prematurely, resulting in fewer cells and thereby fewer neurons produced. Overt toxicity can also result in apoptosis or cell death.

A number of studies report the adverse effects of APAP exposure at the cellular level.

In a study using human neural stem cells as a model for developmental neurotoxicity, APAP was included as one of several chemicals for testing. Background information provided by the study resulted in APAP being initially classified as "non-neurotoxic" with therapeutic concentrations of 33-132μM, and toxic concentrations of 165-992 μM.[134] This study confirmed cytotoxicity at the highest concentration of APAP tested, 1000μM == 1mM.

Studies have shown that the APAP metabolite NAPQI is taken up by mitochondria and depletes ATP (adenosine triphosphate). One of the more dramatic impacts of NAPQI (400μM) on cellular energy production was demonstrated by both the rapid depletion of ATP (>80% depletion with a 1-minute exposure) and almost complete inhibition of oxygen consumption in exposed hepatocytes (~90% inhibition with a 10 second exposure).[135] This concentration is 8-10X concentrations found *in vivo*, but NAPQI also has significant biological impacts at lower concentrations.

For example, NAPQI can bind to Topoisomerase II (Topo II) and interfere with its cellular-nuclear functions.[136] Topo II normally changes the topology of DNA (winding, knotting, tangling strands) by cutting and resealing both strands of DNA, allowing DNA strands to pass through each other. This helps to unknot, untangle, link, or unlink DNA molecules during DNA replication, transcription, and chromosome segregation.[137] It has also been proposed that chromatin packing is triggered by histone-dependent, Topo II-mediated clamping of DNA strands.[138] NAPQI stimulates DNA cutting by Topo II,

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[132] Machnicka et al. The role of spectrin in cell adhesion and cell-cell contact. Exp Biol Med (Maywood). 2019 Nov;244(15):1303-1312. doi: 10.1177/1535370219859003. Epub 2019 Jun 21. Erratum in: Exp Biol Med (Maywood). 2019 Jul 17;:1535370219867051. PMID: 31226892; PMCID: PMC6880153.

[133] Galea et al. Biomechanical coupling facilitates spinal neural tube closure in mouse embryos. Proc Natl Acad Sci U S A. 2017 Jun 27;114(26):E5177-E5186. doi: 10.1073/pnas.1700934114. Epub 2017 Jun 12. PMID: 28607062; PMCID: PMC5495245.

[134] Buzanska et al. A human stem cell-based model for identifying adverse effects of organic and inorganic chemicals on the developing nervous system. Stem Cells. 2009 Oct;27(10):2591-601. doi: 10.1002/stem.179. PMID: 19609937.

[135] Andersson et al. N-acetyl-p-benzoquinone imine-induced changes in the energy metabolism in hepatocytes. Chem Biol Interact. 1990;75(2):201-11. doi: 10.1016/0009-2797(90)90118-7. PMID: 2369786.

[136] Bender et al. N-acetyl-p-benzoquinone imine, the toxic metabolite of acetaminophen, is a topoisomerase II poison. Biochemistry. 2004 Mar 30;43(12):3731-9. doi: 10.1021/bi036107r. PMID: 15035644.

[137] Reviewed in Deweese et al. DNA Topology and Topoisomerases: Teaching a "Knotty" Subject. Biochem Mol Biol Educ. 2008;37(1):2-10. doi: 10.1002/bmb.20244. PMID: 19225573; PMCID: PMC2643378.

[138] Hizume et al. Topoisomerase II, scaffold component, promotes chromatin compaction in vitro in a linker-histone H1-dependent manner. Nucleic Acids Res. 2007;35(8):2787-99. doi: 10.1093/nar/gkm116. Epub 2007 Apr 11. PMID: 17430970; PMCID: PMC1885653.

>5-fold at 100 µM, but repair of the cuts was inhibited by NAPQI.[139] APAP has also shown inhibitory effects on DNA repair in multiple mammalian cells.[140] Cytotoxicity and DNA damage were also examined in hepatoma cells, and results indicated NAPQI produces DNA and cellular damage (Table 4). The cytotoxicity observed at 24hrs with the lowest concentration of NAPQI tested, 0.05mM / 50µM, poses a relevant risk. As indicated below, 1mM APAP is a relevant *in vivo* concentration found in plasma and brains of animals exposed to APAP at therapeutic human equivalent doses.[141] With a concentration of 1mM APAP, then 5-15% would be expected to be oxidized into NAPQI, resulting in NAPQI concentrations of 50-150mM, which produced cytotoxicity.

Test substance		Elution rate constant	Cytotoxicity (% of control)	
			1 h	24 h
DMSO (control)		0.010 ± 0.002	0 ± 0	0 ± 0
Paracetamol	10 mM	0.012 ± 0.004	0 ± 0	0 ± 0
NAPQI	0.05 mM	0.041 ± 0.019	0 ± 0	36 ± 11
	0.10 mM	0.075 ± 0.036	2 ± 2	81 ± 3
	0.25 mM	0.173 ± 0.068	5 ± 1	100 ± 0
N-OH-AAF	0.10 mM	0.155 ± 0.029	0 ± 0	0 ± 0

Table 4. NAPQI Cytotoxicity. Reuber hepatoma cells, prelabelled with 3H-TdR, were exposed to test substances for 1 h. The cultures were then rinsed and prepared for the alkaline elution assay or cytotoxicity was determined immediately or after incubation for an additional 23 h. Means +/- S.D. of 4-8 determinations. APAP does not show cytotoxicity relative to DMSO control. The APAP metabolite NAPQI is cytotoxic and resulted in DNA damage at all tested concentrations (0.05mM-0.25mM) at 24h. [142]

The bulk of APAP toxicity is focused on hepatotoxicity, where APAP has also been shown to produce mitochondrial oxidative and nitrosative stress.[143] The mitochondrial alterations caused by APAP are well-established in the scientific literature and have been recently reviewed.[144] Briefly, the mitochondria are organelles in the cell that produce energy in the form of ATP. The process of producing ATP involves the transfer of electrons from one protein complex to another in the electron transport chain (ETC). During this process, free radicals such as superoxide are generated, but they are normally-efficiently scavenged by superoxide dismutases (SOD) within the mitochondria. However, when mitochondria are exposed to NAPQI, NAPQI can bind with mitochondrial proteins and form "adducts," which are combinations of NAPQI and the protein. Adduct formation on mitochondrial proteins such as ATP synthase can result in reverse electron transport within the electron transport chain, which elevates superoxide generation and induces oxidative damage. Protein adduct formation on antioxidant enzymes prevents efficient scavenging of superoxide, which enables nitric oxide to form peroxynitrite radicals. These radicals can also modify

[139] Bender et al. N-acetyl-p-benzoquinone imine, the toxic metabolite of acetaminophen, is a topoisomerase II poison. Biochemistry. 2004 Mar 30;43(12):3731-9. doi: 10.1021/bi036107r. PMID: 15035644.
[140] Brunborg et al. Inhibitory effects of paracetamol on DNA repair in mammalian cells. Mutat Res. 1995 Apr;342(3-4):157-70. doi: 10.1016/0165-1218(95)90025-x. PMID: 7715617.
[141] Posadas et al. Acetaminophen induces apoptosis in rat cortical neurons. PLoS One. 2010 Dec 10;5(12):e15360. doi: 10.1371/journal.pone.0015360. PMID: 21170329; PMCID: PMC3000821.
[142] Dybing et al. Genotoxicity studies with paracetamol. Mutat Res. 1984 Oct;138(1):21-32. doi: 10.1016/0165-1218(84)90081-8. PMID: 6387477.
[143] Ramachandran and Jaeschke. Acetaminophen hepatotoxicity: A mitochondrial perspective. Adv Pharmacol. 2019;85:195-219. doi: 10.1016/bs.apha.2019.01.007. Epub 2019 Feb 21. PMID: 31307587; PMCID: PMC7350903.
[144] Ramachandran and Jaeschke. A mitochondrial journey through acetaminophen hepatotoxicity. Food Chem Toxicol. 2020 Jun;140:111282. doi: 10.1016/j.fct.2020.111282. Epub 2020 Mar 21. PMID: 32209353; PMCID: PMC7254872.

enzymes such as SOD as well as spill out of the mitochondria into the cytosol resulting in mitochondrial and cytoplasmic interactions and toxicity.

Genetic toxicity was also reported in the NTP carcinogenesis and genetic toxicity studies of APAP. This included negative results in Ames testing using *Salmonella typhimurium*, and positive genetic toxicity findings for sister chromatid exchanges and chromosomal aberrations in Chinese hamster ovary cells.[145] This is in contrast to the leukemias reported by NTP. It was also reported independently by a research group Boehringer Ingelheim Pharmaceuticals that the APAP metabolite (p-Aminophenol, PAP) resulted in reproducible dose-related increases in cells with single-strand DNA breaks with both the mouse lymphoma cells (L5178Y) and the Chinese hamster ovary (CHO) cells.[146]

The damage from NAPQI has been reported to cause apoptosis (cell death) in rat cortical neurons.[147] APAP intoxication has also been reported to produce astrogliosis, an abnormal increase in astrocytes due to destruction of local neurons, in areas of the brain associated with locomotion regulation and to havea direct toxic effect on a mixed primary culture of two types of brain cells, astrocytes and oligodendrocytes.[148] This study also reported that APAP has a direct neurotoxic effect on the selective modulation of dopamine content in motor-regulating regions of the brain due to downregulation of tyrosine hydroxylase expression. This is the first study to provide evidence of a mechanistic link between toxic APAP dosing and direct adverse effects on glial components of the CNS.

In addition, the inhibitory neurons known as Purkinje cells are necessary for well-coordinated movement, cognition, and emotion. They regulate the activation of excitatory neurons by interactions and release gama-aminobutyric acid (GABA), a neurotransmitter that inhibits certain neurons from transmitting impulses. These cells are vulnerable to oxidative stress, and some forms of autism have significant loss of Purkinje cells in the cerebellum.[149]

APAP Disrupts the Endocannabinoid Signaling Essential for Brain Development.

The endocannabinoid cell-signaling system (ECS) includes cannabinoid receptors CB1 (CB1R) and CB2 (CB2R), the endocannabinoid agonists (activators) anandamide (AEA) and 2-arachidonoylglycerol (2-AG), and the enzymes that synthesize and metabolize the endocannabinoid ligands.[150]

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[145] National Toxicology Program. NTP Toxicology and Carcinogenesis Studies of Acetaminophen (CAS No. 103-90-2) in F344 Rats and B6C3F1 Mice (Feed Studies). Natl Toxicol Program Tech Rep Ser. 1993 Jan;394:1-274. PMID: 12637965.

[146] Majeska and Holden. Genotoxic effects of p-aminophenol in Chinese hamster ovary and mouse lymphoma cells: results of a multiple endpoint test. Environ Mol Mutagen. 1995;26(2):163-70. doi: 10.1002/em.2850260210. PMID: 7556113.

[147] Posadas I, Santos P, Blanco A, Muñoz-Fernández M, Ceña V. Acetaminophen induces apoptosis in rat cortical neurons. PLoS One. 2010 Dec 10;5(12):e15360. Doi:10.1371/ journal.pone.0015360.

[148] Vigo et al. Acute acetaminophen intoxication induces direct neurotoxicity in rats manifested as astrogliosis and decreased dopaminergic markers in brain areas associated with locomotor regulation. Biochem Pharmacol. 2019 Dec;170:113662. doi: 10.1016/j.bcp.2019.113662. Epub 2019 Oct 10. PMID: 31606411.

[149] Tamiji and Crawford. The neurobiology of lipid metabolism in autism spectrum disorders. Neurosignals. 2010;18(2):98-112. doi: 10.1159/000323189. Epub 2011 Feb 4. PMID: 21346377.

[150] Maccarroneet al. Endocannabinoid signaling at the periphery: 50 years after THC. Trends Pharmacol Sci. 2015 May;36(5):277-96. doi: 10.1016/j.tips.2015.02.008. Epub 2015 Mar 18. PMID: 25796370; PMCID: PMC4420685.

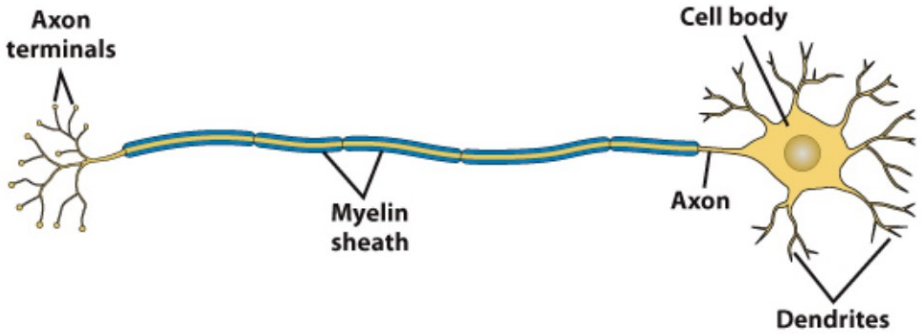

Figure 14. Schematic diagram of an idealized neuron with major components.

Neurons come in a variety of types, such as serotonergic or dopaminergic, but are collectively specialized cells that transmit information throughout the nervous system. They have a cell body and branching structures called dendrites and axons (Figure 14). Dendrites receive signals from other neurons, while axons send signals to other neurons. The point where an axon terminal of one neuron meets the dendrite of another neuron is called a synapse. Neuronal signaling occurs when an electrical signal travels from the cell body along the axon to the axon terminal, where it triggers the release of neurotransmitters. These neurotransmitters cross the synaptic cleft and bind to receptors on the dendrite of the next neuron, transmitting the signal. There are more than 100 known neuropeptide neurotransmitters in the human nervous system.[151] These include glutamate, GABA (gamma-aminobutyric acid), acetylcholine, glycine, and norepinephrine, dopamine, serotonin, histamine, and endocannabinoids. Endocannabinoids are reported to regulate a variety of physiological processes, including cardiovascular, gastrointestinal, and hepatic (liver) functions, adaptive and innate immunity, muscle formation, bone remodeling, pain sensation, mood, and memory.[150]

The endocannabinoid cell-signaling system (ECS) targets two receptors known as the CB1 and CB2 cannabinoid receptors. The neurotransmitter molecule that is the activator (or agonist) for the CB1 receptor is anandamide (an endogenous cannabinoid). The activator/agonist for the CB2 receptor is 2-arachidonoylglycerol (2-AG).[152] Studies have also suggested the occurrence of other endocannabinoid targets beyond CB1 and CB2, proposing GPR55 and GPR35 share common interactions through a metabolite of 2-AG, 2-arachidonoyl lysophosphatidic acid (LPA).[153]

APAP is reported to cause CB1 and CB2 activation through several mechanisms. APAP can be metabolized via hydrolysis to form p-aminophenol (PAP). Then PAP undergoes conjugation with arachidonic acid, using the enzyme fatty acid amid hydrolase (FAAH), to produce N-acylphenolamine

[151] Cuevas. Neurotransmitters and their Life Cycle. xPharm: The Comprehensive Pharmacology Reference. 1-7. 2011. doi: 10.1016/B978-008055232-3.60016-9.
[152] Maccarroneet al. Endocannabinoid signaling at the periphery: 50 years after THC. Trends Pharmacol Sci. 2015 May;36(5):277-96. doi: 10.1016/j.tips.2015.02.008. Epub 2015 Mar 18. PMID: 25796370; PMCID: PMC4420685.
[153] Zhao and Abood. GPR55 and GPR35 and their relationship to cannabinoid and lysophospholipid receptors. Life Sci. 2013 Mar 19;92(8-9):453-7. doi: 10.1016/j.lfs.2012.06.039. Epub 2012 Jul 20. PMID: 22820167.

(AM404).[154] The AM404 metabolite increases brain endocannabinoid levels by decreasing the re-uptake of anandamide. The result is an increased level of anandamide.[155] In addition, the chemical structure of AM404 is similar to anandamide (Figure 15).[156] Because of these similarities, AM404 can bind with and activate the CB1 receptor (although it is a weaker agonist than anandamide).[155] AM404 is also reported be an agonist (activator) of the CB2 receptor.[157] CB2 is expressed in peripheral and immune tissues, and animal studies have detected it in various brain regions, including olfactory tubercle, islands of Calleja, cerebral cortex, striatum, thalamic nuclei, hippocampus, amygdala, substantia nigra, periaqueductal gray, paratrochlear nucleus, paralemniscal nucleus, red nucleus, pontine nuclei, inferior colliculus and the parvocellular portion of the medial vestibular nucleus.[158]

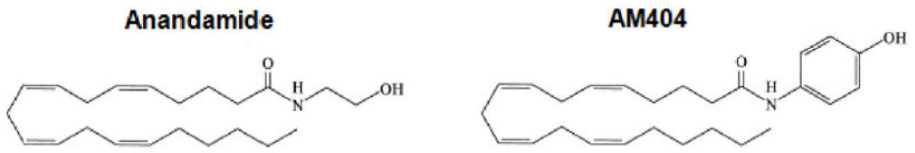

Figure 15. Structures of Endocannabinoid Anandamide and Acetaminophen Metabolite AM404. Both molecules share a common 20-carbon backbone derived from arachidonic acid.

At typical therapeutic doses, APAP and/or the metabolite AM404 mediate endocannabinoid signaling. This effect is considered to be a primary mechanism of action by which APAP relieves pain.[155] For example, experiments have demonstrated that disrupting the CB1 receptor in mice or rats (either genetically or through pharmacological antagonist) results in loss of the analgesic activity of APAP.[159,155] In addition, inhibition of FAAH will suppress the analgesic effect of acetaminophen, which indicates that acetaminophen's pain modification depends on production of AM404 which makes use of FAAH.[159] In one notable case study, a Scottish woman who felt no pain was reported to lack the gene that produces FAAH, clearly demonstrating the analgesic impact of this molecular pathway.[160]

While these pharmacological effects on the endocannabinoid system are useful in adults as a temporary analgesic or antipyretic, this same pathway is essential for brain development, and disruption of it during

[154] Högestätt et al. Conversion of acetaminophen to the bioactive N-acylphenolamine AM404 via fatty acid amide hydrolase-dependent arachidonic acid conjugation in the nervous system. J Biol Chem. 2005 Sep 9;280(36):31405-12. doi: 10.1074/jbc.M501489200. Epub 2005 Jun 29. PMID: 15987694.
[155] Bertolini, A., Ferrari, A., Ottani, A., Guerzoni, S. Tacchi, R., Leone, S., 2006, paracetamol: new vistas of an old drug. CNS Drug Rev. 12 (3-4), 250-275, p. 255.
[156] Ghanen, C., Perez Mariz, J., Manautou, J., Mottino, A., Acetaminiophen; From liver to brain: new insights into drug pharmacological action and toxicity, Pharmacol Res. 2016 July; 109: 119-131, DOI: 10.1016/ j.phrs.2016.02.020, p. 3.
[157] Buhrer C, Endesfelder S, Scheuer T, Schmitz T, Paracetamol (Acetaminophen) and the Developing Brain, Int. J. Mol. Sci. 2021, 22, 11156, DOI: 10.3390/ ijms222011156, p.4.
[158] Gong et al. Cannabinoid CB2 receptors: immunohistochemical localization in rat brain. Brain Res. 2006 Feb 3;1071(1):10-23. doi: 10.1016/j.brainres.2005.11.035. Epub 2006 Feb 9. PMID: 16472786.
[159] Mallet et al. Endocannabinoid and serotonergic systems are needed for acetaminophen-induced analgesia. Pain. 2008 Sep 30;139(1):190-200. doi: 10.1016/j.pain.2008.03.030. Epub 2008 May 15. PMID: 18485596.
[160] Habib et al. Microdeletion in a FAAH pseudogene identified in a patient with high anandamide concentrations and pain insensitivity. Br J Anaesth. 2019 Aug;123(2):e249-e253. doi: 10.1016/j.bja.2019.02.019. Epub 2019 Mar 28. PMID: 30929760; PMCID: PMC6676009.

critical windows of brain development can thereby disrupt neurodevelopment. This is because the endocannabinoid system is required for normal brain development.[161]

Endocannabinoids regulate synaptogenesis, axon growth and positioning, and neuronal cell fate.[162] They also regulate both the number and division rate of stem cells that differentiate into neural cells and the type of neuronal cell (neuron or glia) produced from neural stem cells.[161] Endocannabinoids also guide the migration of the neural cells "to their final positions in the cerebral cortex and other brain areas..." and after the cells have arrived at their final location, endocannabinoid molecules affect how the cells build neuronal networks. [162]

It has also been reported that there is a transient window when the dominant brain cannabinoid receptor, CB1, is expressed on afferent terminals (the nerve fibers that extend from the brain to sensory receptors such as on the skin) instead of on the Purkinje neuron cell synapses that dominate the adult cerebellum. The activation of these afferent CB1 receptors during development suppresses synaptic transmission onto developing granule cells (the most common type of neuron in the brain) and consequently suppresses excitation of downstream neurons in the developing cortical network, including nonsynaptic, migrating neurons.[163] This study also reports:

> …there is a transient period in the newborn rodent, during the cerebellar "growth spurt," when functional CB1Rs are expressed on MF (mossy fiber) afferent terminals and on inhibitory interneuron cross-connected synapses, but not on the excitatory or inhibitory afferents to PCs (Purkinje cells) that dominate in the adult... (inserted)

A direct analogy can be made between fetal exposure to APAP and fetal exposure to marijuana. This is supported by evidence that phyto-based (plant) cannabinoids, such as delta-9-tetrahydrocannabinol (Δ9THC), are reported to have an impact on neurodevelopment similar to that seen in exposure to APAP. Exposing mice to THC on post-natal day 10 (corresponding to end of third-trimester exposure in humans, at peak brain growth spurt) results in altered spontaneous behavior and habituation rates in adult mice.[164] Similar behavioral alterations occur when mice are exposed to APAP on PND10.[165, 166] Because THC and APAP both interact with the endocannabinoid system, it has been hypothesized that this system might be involved in the same adverse outcome pathway for both of these drugs.[167]

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[161] Harkany and Cinquina. Physiological Rules of Endocannabinoid Action During Fetal and Neonatal Brain Development. Cannabis Cannabinoid Res. 2021 Oct;6(5):381-388. doi: 10.1089/can.2021.0096. Epub 2021 Oct 6. PMID: 34619043; PMCID: PMC8664114.

[162] Gaffuri et al. Type-1 cannabinoid receptor signaling in neuronal development. Pharmacology. 2012;90(1-2):19-39. doi: 10.1159/000339075. Epub 2012 Jul 3. PMID: 22776780.

[163] Barnes et al. Developmentally Transient CB1Rs on Cerebellar Afferents Suppress Afferent Input, Downstream Synaptic Excitation, and Signaling to Migrating Neurons. J Neurosci. 2020 Aug 5;40(32):6133-6145. doi: 10.1523/JNEUROSCI.1931-19.2020. Epub 2020 Jul 6. PMID: 32631938; PMCID: PMC7406284.

[164] Philippott, G., Forsberg, E., Tahan, C., Viberg, H., & Fredriksson, R. A single Δ9-Tetrahyddrocannabinol THC) dose during brain development affects markers of neurotrophy, oxidative stress, and apoptosis (2019) Front. Pharmacol. 10:1156, DOI:10.3389/ fphar.2019.01156

[165] Viberg et al. Paracetamol (acetaminophen) administration during neonatal brain development affects cognitive function and alters its analgesic and anxiolytic response in adult male mice. Toxicol Sci. 2014 Mar;138(1):139-47. doi: 10.1093/toxsci/kft329. Epub 2013 Dec 21. PMID: 24361869.

[166] Philippot et al. Adult neurobehavioral alterations in male and female mice following developmental exposure to paracetamol (acetaminophen): characterization of a critical period. J Appl Toxicol. 2017 Oct;37(10):1174-1181. doi: 10.1002/jat.3473. Epub 2017 Apr 27. PMID: 28448685.

[167] Philippott, G., Forsberg, E., Tahan, C., Viberg, H., & Fredriksson, R. A single Δ9-Tetrahyddrocannabinol THC) dose during brain development affects markers of neurotrophy, oxidative stress, and apoptosis (2019) Front. Pharmacol. 10:1156, DOI:10.3389/ fphar.2019.01156

As indicated above, the chemical structures of the APAP metabolite AM404 are built on the same backbone as the endocannabinoid, anandamide. While Δ9THC lacks this backbone, it does bind with the same receptors as anandamide, including CB1.[168] Rodent animal studies report that even low doses of cannabinoid compounds can result in atypical locomotor activity, cognitive impairments, altered emotional behavior and clinical studies support this interaction.[169] There were similar findings in children with prenatal *in utero* exposure to cannabis (Δ9THC) that have prompted public health announcements (Figure 16). Reported neurobehavioral abnormalities include increased hyperactivity, impulsivity, and inattention symptoms,[170] First trimester exposure also predicted deficits in Wide Range Achievement Test-Revised (WRAT-R) reading and spelling scores and a lower rating on the teachers' evaluations of the children's performance, and second-trimester marijuana use was significantly associated with reading comprehension and underachievement,[171] altered executive functions including attentional behavior,[172] and altered neural functioning during visuospatial working memory processing.[173] Several studies have reported that *in utero* exposure to cannabis is associated and with ASD[174] and with ADHD.[175]

[168] https://nida.nih.gov/publications/research-reports/marijuana/how-does-marijuana-produce-its-effects.

[169] Campolongo et al. Developmental consequences of perinatal cannabis exposure: behavioral and neuroendocrine effects in adult rodents. Psychopharmacology (Berl). 2011 Mar;214(1):5-15. doi: 10.1007/s00213-010-1892-x. Epub 2010 Jun 17. PMID: 20556598; PMCID: PMC3045519.

[170] Goldschmidt et al. Effects of prenatal marijuana exposure on child behavior problems at age 10. Neurotoxicol Teratol. 2000 May-Jun;22(3):325-36. doi: 10.1016/s0892-0362(00)00066-0. PMID: 10840176.

[171] Goldschmidt et al. Prenatal marijuana and alcohol exposure and academic achievement at age 10. Neurotoxicol Teratol. 2004 Jul-Aug;26(4):521-32. doi: 10.1016/j.ntt.2004.04.003. PMID: 15203174.

[172] Fried and Smith. A literature review of the consequences of prenatal marihuana exposure. An emerging theme of a deficiency in aspects of executive function. Neurotoxicol Teratol. 2001 Jan-Feb;23(1):1-11. doi: 10.1016/s0892-0362(00)00119-7. PMID: 11274871.

[173] Smith et al. Effects of prenatal marijuana on visuospatial working memory: an fMRI study in young adults. Neurotoxicol Teratol. 2006 Mar-Apr;28(2):286-95. doi: 10.1016/j.ntt.2005.12.008. Epub 2006 Feb 13. PMID: 16473495.

[174] Corsi et al. Maternal cannabis use in pregnancy and child neurodevelopmental outcomes. Nat Med. 2020 Oct;26(10):1536-1540. doi: 10.1038/s41591-020-1002-5. Epub 2020 Aug 10. PMID: 32778828; DiGuiseppi et al. Peri-Pregnancy Cannabis Use and Autism Spectrum Disorder in the Offspring: Findings from the Study to Explore Early Development. J Autism Dev Disord. 2022 Nov;52(11):5064-5071. doi: 10.1007/s10803-021-05339-4. Epub 2021 Nov 12. PMID: 34767135; PMCID: PMC9112286; Nutor et al. Prenatal Cannabis Use and Offspring Autism-Related Behaviors: Examining Maternal Stress as a Moderator in a Black American Cohort. J Autism Dev Disord. 2023 Apr 25:1–13. doi: 10.1007/s10803-023-05982-z. Epub ahead of print. PMID: 37097527; PMCID: PMC10127191.

[175] Corsi et al. Maternal cannabis use in pregnancy and child neurodevelopmental outcomes. Nat Med. 2020 Oct;26(10):1536-1540. doi: 10.1038/s41591-020-1002-5. Epub 2020 Aug 10. PMID: 32778828; Smith A, Kaufman F, Sandy MS, Cardenas A. Cannabis Exposure During Critical Windows of Development: Epigenetic and Molecular Pathways Implicated in Neuropsychiatric Disease. Curr Environ Health Rep. 2020 Sep;7(3):325-342. doi: 10.1007/s40572-020-00275-4. PMID: 32441004; PMCID: PMC7458902; McLemore and Richardson. Data from three prospective longitudinal human cohorts of prenatal marijuana exposure and offspring outcomes from the fetal period through young adulthood. Data Brief. 2016 Oct 18;9:753-757. doi: 10.1016/j.dib.2016.10.005. PMID: 27833935; PMCID: PMC5096595; Cioffredi LA, Anderson H, Loso H, East J, Nguyen P, Garavan H, Potter A. Prenatal cannabis exposure predicts attention problems, without changes on fMRI in adolescents. Neurotoxicol Teratol. 2022 May-Jun;91:107089. doi: 10.1016/j.ntt.2022.107089. Epub 2022 Mar 18. PMID: 35314358; PMCID: PMC9136933; Tchuente et al. Is in-utero exposure to cannabis associated with the risk of attention deficit with or without hyperactivity disorder? A cohort study within the Quebec Pregnancy Cohort. BMJ Open. 2022 Aug 8;12(8):e052220. doi: 10.1136/bmjopen-2021-052220. PMID: 35940828; PMCID: PMC9364390.

Marijuana
AND
Pregnancy
If you use marijuana during pregnancy, you may be putting your health and your fetus's health at risk.
Possible Effects on Your Fetus
Disruption of brain development before birth
Smaller size at birth
Higher risk of stillbirth
Higher chance of being born too early, especially when a woman uses both marijuana and cigarettes during pregnancy
Harm from secondhand marijuana smoke
Behavioral problems in childhood and trouble paying attention in school
Possible Effects on You
Permanent lung injury from smoking marijuana
Dizziness, putting you at risk of falls
Impaired judgment, putting you at risk of injury
Lower levels of oxygen in the body, which can lead to breathing problems

Figure 16. Marijuana and Pregnancy. The American College of Obstetricians and Gynecologists warns that using marijuana during pregnancy may affect the fetus by "Disruption of brain development before birth."[176] Marijuana/cannabis contain Δ9THC, which impacts the same pathway as AM404, an APAP metabolite.

APAP disruption of prostaglandins during brain development.

As discussed above, APAP inhibits synthesis of prostaglandins.[177] The impact of APAP on prostaglandins, which mediate the generation of fevers, has been proposed as one of the mechanisms for the anti-pyretic action of APAP.[178] The inhibition of prostaglandins in an adult can be beneficial to the extent it modulates fever or pain, as reviewed above. Unfortunately, inhibiting prostaglandins during brain development disrupts multiple aspects of neural development that are prostaglandin dependent. The prostaglandin E2 is used in neurodevelopment to signal the formation of dendritic spines (the branch-like structures on the

[176] https://www.acog.org/womens-health/infographics/marijuana-and-pregnancy
[177] Flower and Vane. Inhibition of prostaglandin synthetase in brain explains the anti-pyretic activity of paracetamol (4-acetamidophenol). Nature (1972) Dec 15;240(5381):410-1. doi: 10.1038/240410a0. PMID: 4564318.; Mitchell et al. Selectivity of nonsteroidal antiinflammatory drugs as inhibitors of constitutive and inducible cyclooxygenase (1993) Proc Natl Acad Sci USA; 90(24):11693-7. DOI:10.1073/pnas.90.24.11693. PMID: 8265610; PMCID: PMC48050.
[178] Bertolini et al. Paracetamol: new vistas of an old drug. CNS Drug Rev. 2006 Fall-Winter;12(3-4):250-75. doi: 10.1111/j.1527-3458.2006.00250.x. PMID: 17227290; PMCID: PMC6506194.

cell body of a neuron, (Figure 14).[179] In addition, prostaglandins are necessary during brain development, influencing both the arborization and pruning of immature or inappropriate excitatory connections. Exposing rats to APAP near the peak of the brain growth spurt (BGS) at doses clinically relevant to humans was shown to disrupt prostaglandin E2 synthesis and the development of Purkinje neuron cells in the cerebellum.[180] This exposure initially increased dendritic growth, followed by cerebellar atrophy, which then resulted in altered social interactions.

APAP impacts on vanilloid and serotonergic pathways

The APAP metabolite AM404 is a "potent activator of vanilloid subtype 1 receptors (TRPV1)."[181] In the brain, TRPV1 receptors are found on neurons, as well as in microglia and astrocytes. Microglia are cells that form the main active immune system for the central nervous system.[182] Astrocytes are a type of glial cell that perform numerous functions, including providing nutrients to neural cells, maintaining ion balance, regulating cerebral blood flow, and maintaining the brain-blood barrier. One function of TRPV1 signaling is to activate inflammatory responses in the brain, and dysfunction of TRPV1 is involved in the development of immune-mediated neurological disorders in the central nervous system.[183] The inflammatory oxidative stress from TRPV1 activation may also contribute synergistically to the oxidative stress caused by the APAP metabolite NAPQI.

The transient receptor potential vanilloid 1 (TRPV1) ion channel is also reported to function in endocannabinoid signaling in microglia, the innate immune cells of the central nervous system (CNS).[184] TRPV1 is a ligand-gated and nonselective cationic channel that is activated by capsaicin (the "heat" molecule in some peppers) and actual heat.[185] In the last few years, a number of studies have suggested a physiological role for AEA as a TRPV1 agonist.[186] The interaction of AEA with TRPV1 and/or CB1 mediates cell death of dopaminergic neurons via cytosolic binding, triggering activation of nonselective ion channels, activation of protein kinases, and increases in intracellular Ca++ concentration, and can result in mitochondrial uncoupling and cell death.[187]

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[179] Tamiji J, Crawford DA. The neurobiology of lipid metabolism in autism spectrum disorders. Neurosignals. 2010;18(2):98-112. doi: 10.1159/000323189. Epub 2011 Feb 4. PMID: 21346377

[180] Dean et al. Prostaglandin E2 is an endogenous modulator of cerebellar development and complex behavior during a sensitive postnatal period. Eur J Neurosci. 2012 Apr;35(8):1218-29. doi: 10.1111/j.1460-9568.2012.08032.x. PMID: 22512254; PMCID: PMC3534986.

[181] Bertolini et al. Paracetamol: new vistas of an old drug. CNS Drug Rev. 2006 Fall-Winter;12(3-4):250-75. doi: 10.1111/j.1527-3458.2006.00250.x. PMID: 17227290; PMCID: PMC6506194.

[182] Bertolini et al. Paracetamol: new vistas of an old drug. CNS Drug Rev. 2006 Fall-Winter;12(3-4):250-75. doi: 10.1111/j.1527-3458.2006.00250.x. PMID: 17227290; PMCID: PMC6506194.

[183] Kong, W., Peng Y., Peng B. (2017), Modulation of neuroinflammation: Role and therapeutic potential of TRPV1 in the neuro-immune axis, Brain, Behavior, and Immunity, 64:354-366, DOI: 10.1016/ j.bbi.2017.03.007.

[184] Marinelli Set al. Endocannabinoid signaling in microglia. Glia. 2023 Jan;71(1):71-90. doi: 10.1002/glia.24281. Epub 2022 Oct 12. PMID: 36222019.

[185] Szallasi and Blumberg. Vanilloid (Capsaicin) receptors and mechanisms. Pharmacol Rev. 1999 Jun;51(2):159-212. PMID: 10353985.

[186] De Petrocellis et al. The activity of anandamide at vanilloid VR1 receptors requires facilitated transport across the cell membrane and is limited by intracellular metabolism. J Biol Chem. 2001 Apr 20;276(16):12856-63. doi: 10.1074/jbc.M008555200. Epub 2001 Jan 26. PMID: 11278420.

[187] Kim et al. Transient receptor potential vanilloid subtype 1 mediates cell death of mesencephalic dopaminergic neurons in vivo and in vitro. J Neurosci. 2005 Jan 19;25(3):662-71. doi: 10.1523/JNEUROSCI.4166-04.2005. PMID: 15659603; PMCID: PMC6725326.

APAP also has indirect effects on serotonergic levels and signaling.[188,189,190] Serotonin signaling facilitates neurogenesis, cell migration, synaptogenesis, and synaptic plasticity.[191] In addition, serotonin activation causes vasoconstriction, which reduces blood flow and restricts the exchange of oxygen, nutrients, and waste products.

APAP impacts neural crest cell arborization

Altered structure and communication pathways that occur during early development are hallmarks of ADHD and ASD, as reported in functional and imaging studies of animals and humans (described below). A 2022 study reported on the effects of APAP in altering neuron structures and pathways.[192] The researchers exposed human neurons (NT2N) and chicken neurons (CGN) to APAP at various concentrations and for various durations. Chicken neurons were used in addition to human cells to test the robustness of the results. If the same cause and effect are replicated in cells from different species, it is unlikely that the observed effects were an artifact of the particular human cells used, and more likely that the effects reflect chemical and cellular mechanisms common to all neurons. APAP was reported to disrupt the cytoskeletal proteins SPTBN1 (Figure 17) and TUBB3 in neurons in both human (Figure 17, bottom) and avian (Figure 17, top) cell culture. The left column depicts the controls (Ctrl) and provide a reference for normal *in vitro* growth of structural proteins in neurons that are not exposed to APAP. The next two columns depict the results of neuronal cells exposed to APAP. There is evidence of decreased structural proteins and loss of entire neuronal cells and networks with increasing APAP exposure.

Quantitative analysis of these experiments indicates that APAP disrupted neuronal arborization or branching (Figure 18). Neurons exposed to APAP had a reduction in the number of branch points and a reduction in the length of neurites. The analogy is trees (neurons) with smaller and fewer branches. The effect increased with exposure to higher concentrations of APAP and with exposure to APAP for longer durations.

[188] Karandikar et al. Effect of drugs modulating serotonergic system on the analgesic action of paracetamol in mice. Indian J Pharmacol. 2016 May-Jun;48(3):281-5. doi: 10.4103/0253-7613.182874. PMID: 27298498; PMCID: PMC4900001.
[189] Pelissier et al. Paracetamol exerts a spinal antinociceptive effect involving an indirect interaction with 5-hydroxytryptamine3 receptors: in vivo and in vitro evidence. J Pharmacol Exp Ther. 1996 Jul;278(1):8-14. PMID: 8764329.
[190] Dogrul et al. Systemic paracetamol-induced analgesic and antihyperalgesic effects through activation of descending serotonergic pathways involving spinal 5-HT_7 receptors. Eur J Pharmacol. 2012 Feb 29;677(1-3):93-101. doi: 10.1016/j.ejphar.2011.12.016. Epub 2011 Dec 21. PMID: 22206817.
[191] Eissa, N., Al-Hougani, M., Sadeq, A., Ojha, SK., Sasses, A., Sadek, B. (2018) Current enlightenment about etiology and pharmacological treatment of autism spectrum disorder, Frontiers in Neurosicence, 12:304, DOI: 10.3389/ fnins.2018.00304, p.2.
[192] Labba et al. Paracetamol perturbs neuronal arborization and disrupts the cytoskeletal proteins SPTBN1 and TUBB3 in both human and chicken in vitro models. Toxicol Appl Pharmacol. 2022 Aug 15;449:116130. doi: 10.1016/j.taap.2022.116130. Epub 2022 Jun 15. PMID: 35714712.

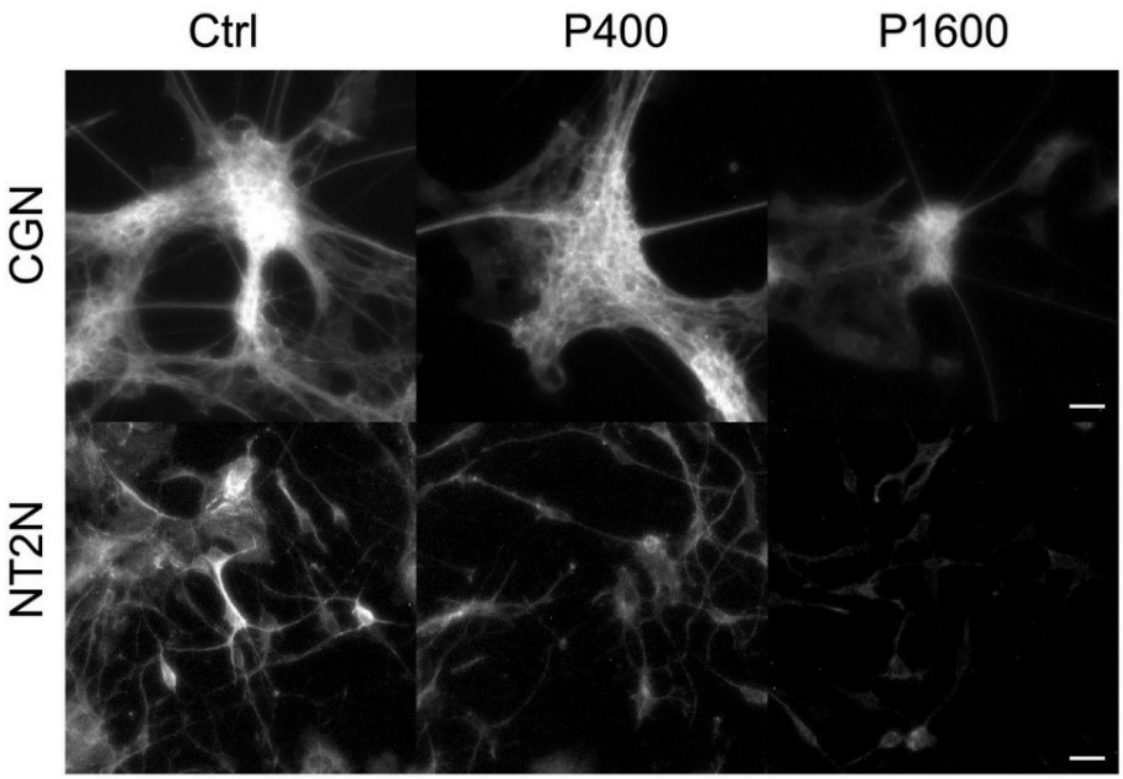

Figure 17. Changes in Neuronal Structural Protein (Spectrin). Fluorescent images of SPTBN1 in control samples of chicken CGNs (top row) and human NT2Ns (bottom row), exposed to increasing concentrations of APAP. Images were taken at 20× magnification, scale bar = 50 µm.[193]

[193] Labba et al. Paracetamol perturbs neuronal arborization and disrupts the cytoskeletal proteins SPTBN1 and TUBB3 in both human and chicken in vitro models. Toxicol Appl Pharmacol. 2022 Aug 15;449:116130. doi: 10.1016/j.taap.2022.116130. Epub 2022 Jun 15. PMID: 35714712.

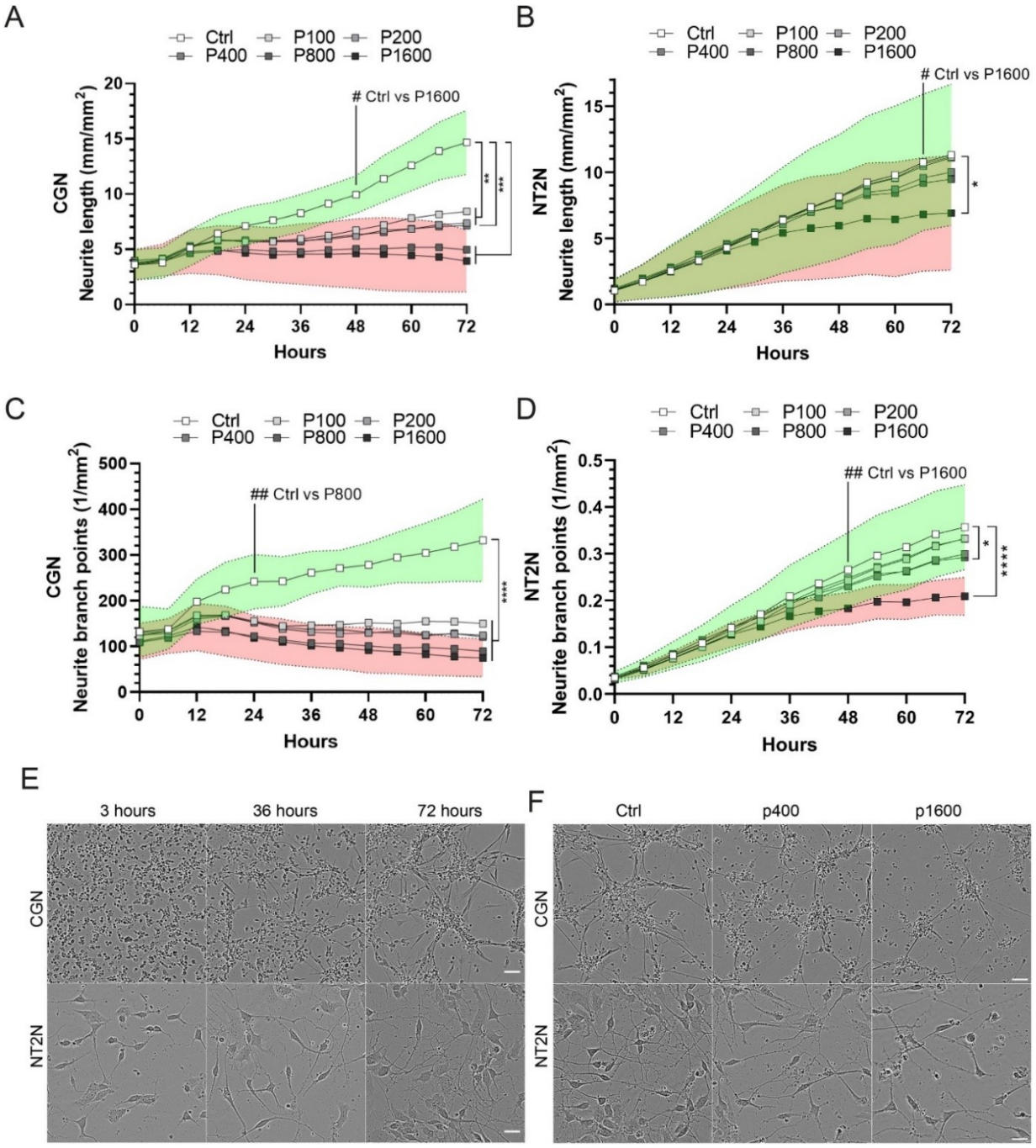

Figure 18. The Effects of Acetaminophen (APAP) on Neurite Arborization in CGNs and NT2N. Cells were exposed to 100–1600 µM APAP for up to 72 h and imaged. The images were used to quantify the neurite lengths of (A) chicken CGNs and (B) human NT2Ns, as well as the neurite branch of points of (C) CGNs and (D) NT2Ns. (E) Representative phase contrast images of CGNs (top row) and NT2Ns (bottom row) from 3 h (first column), 36 h (middle column), and 72 h (last column). (F) Phase contrast images of CGNs (top row) and NT2Ns (bottom row) showing control samples (first column), and samples exposed to APAP at 400 µM (middle column), and 1600 µM (last column). Asterisks denote significance, Dunnett's MCT, $\alpha = 0.05$, ctrl vs sample. Hashes denote first LOAEL, multiples = higher significance. All experiments were performed in biological triplicate, nCGN = 4, nNT2N = 12, 4 images per well. Images were taken at 10× magnification. Data are shown as means, with variance shown as ± SD for controls (green) and P1600 (red).

The Labba study supports that APAP can have harmful effects on the development of the brain and nervous system.[194] Mechanistically, these reported impacts on neurons are consistent with interactions with cannabinoid signaling. Specifically, cannabinoid signaling participates in the modulation of neuronal and glial proliferation. The influence of the APAP metabolite AM404 is also reported to alter endocannabinoid anandamide signaling and can thereby also alter neural cell proliferation. Anandamide also influences embryogenesis, where it is reported to induce a variety of neurological and biological effects, such as the differentiation of neuronal progenitor cells.[195]

PAP, Anandamide, and Apoptosis in Cortical Neurons

A study by Schultz et al. investigated the effects of APAP and its metabolite PAP on the viability of mouse-cultured cortical neurons.[196] The authors hypothesized that PAP, which can be metabolized into AM404, see Acetaminophen Metabolism above, and acts as an indirect agonist of cannabinoid receptors, would cause neuronal apoptosis similar to the endocannabinoid anandamide. The test system used in this study was primary cortical neurons prepared from C57BL/6 mouse embryos at embryonic day 15. The control treatments were culture medium with or without ethanol, which was used as a solvent for some of the drugs. The study design was an experimental one, where the neurons were exposed *in vitro* to various concentrations of APAP, PAP, or anandamide for 24 or 48 hours. The statistical methods used were analysis of variance (ANOVA) with Neuman–Keuls post hoc test and two-sample t-test to assess differences between groups. The reported endpoints were cell viability, measured by counting cells labelled with fluorescein diacetate and propidium iodide (live/dead assay) via fluorescent imaging. The conclusions were that PAP and anandamide caused significant loss of neuronal viability at concentrations down to 1 μg/ml, while APAP had no toxic effect at therapeutic doses. The authors suggested that PAP is acting through cannabinoid receptors to induce neuronal apoptosis, and that this has implications for the safety of APAP use in children or individuals with brain injury. According to the authors of the report,

"We are suggesting that the abnormal brain development seen in autism may be due to the use of acetaminophen at a critical early age."

As indicated above and in the following sections, the APAP metabolites, including NAPQI, PAP, and AM404, have multiple-related influences on cell behavior and are thereby proposed and reported to disrupt neurodevelopmental cascades via oxidative stress, oxidative damage, and disruption of endocannabinoid signaling.

[194] Labba et al. Paracetamol perturbs neuronal arborization and disrupts the cytoskeletal proteins SPTBN1 and TUBB3 in both human and chicken in vitro models. Toxicol Appl Pharmacol. 2022 Aug 15;449:116130. doi: 10.1016/j.taap.2022.116130. Epub 2022 Jun 15. PMID: 35714712.
[195] Ruiz-Contreras et al. Modulatory Activity of the Endocannabinoid System in the Development and Proliferation of Cells in the CNS. Neurotox Res. 2022 Dec;40(6):1690-1706. doi: 10.1007/s12640-022-00592-6. Epub 2022 Dec 16. PMID: 36522511.
[196] Schultz et al. Effects of the analgesic acetaminophen (Paracetamol) and its para-aminophenol metabolite on viability of mouse-cultured cortical neurons. Basic Clin Pharmacol Toxicol. 2012 Feb;110(2):141-4. doi: 10.1111/j.1742-7843.2011.00767.x. Epub 2011 Aug 20. PMID: 21771276.

3. Tissue Interactions

Acetaminophen and key metabolites of APAP reach the developing brain, cause changes consistent with the AOP, and result in ASD and ADHD.

i. Increased APAP Concentrations in Fetal Rat Cerebral Spinal Fluid and Brain

Acetaminophen ingested during pregnancy reaches the brain of the fetus. Once APAP is in maternal circulation, it readily passes through the placenta into fetal circulation. For example, in one study 34 pregnant women were given a single dose of APAP upon admission for C-section.[197] Maternal and fetal blood samples were taken at the time of deliver (30 minutes after administering the APAP). The fetal and maternal concentrations of APAP were comparable (11.2μg/mL fetal, 12.3 μg/mL maternal Cmax), which demonstrates that APAP readily crosses the placenta. APAP crosses the placenta and enters fetal blood circulation through the umbilical vein. A unique anatomical feature of the fetal circulatory system called the ductus venosus allows a portion of the highly oxygenated blood from the placenta to bypass the liver and flow directly into the inferior vena cava. From there, the blood is directed towards the right atrium of the fetal heart.[198] This bypass allows APAP to avoid liver metabolism in the fetus, cross the blood brain barrier, and reach the fetal brain.[199] This shunting also limits the ability of the fetal liver to metabolize APAP as it enters fetal circulation, as demonstrated by APAP treatment of Nrf2 mutant mice.[200] Two-thirds of these mutants have congenital intrahepatic shunts, modeling patent ductus venosus, and those mutants with shunts are reported to have diminished hepatoxicity due to APAP exposures (250mg/Kg).

In order to determine APAP exposure levels in the developing brain, Koehn et al. performed a study on rats at three different developmental stages (adults, postnatal day 4 (P4), and gestational day 19 (E19)). They reported **APAP concentrations were two to seven times higher in developing brains compared to adults** (Figure 19, A) and that APAP accumulated in the developing brain in higher amounts than the adult brain. Considering that the adult human therapeutic dose is 1g, and not more than 4g over 24 hours, and the toxic dose is 7g over 24 hours, a seven-fold higher exposure in a developing brain at 2.5hrs is of grave concern regarding embryonic or fetal developmental toxicity. This study also found that chronic treatment of acetaminophen increased this transfer into the fetal brain even more (Figure 19, B), suggesting that the developing brain may be at greater risk from chronic exposure to APAP.

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[197] Nitsche et al. Transplacental Passage of Acetaminophen in Term Pregnancy. Am J Perinatol. 2017 May;34(6):541-543. doi: 10.1055/s-0036-1593845. Epub 2016 Nov 2. PMID: 27806383.

[198] Remien and Majmundar. Physiology, Fetal Circulation. 2023 Apr 26. In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing; 2023 Jan–. PMID: 30969532.

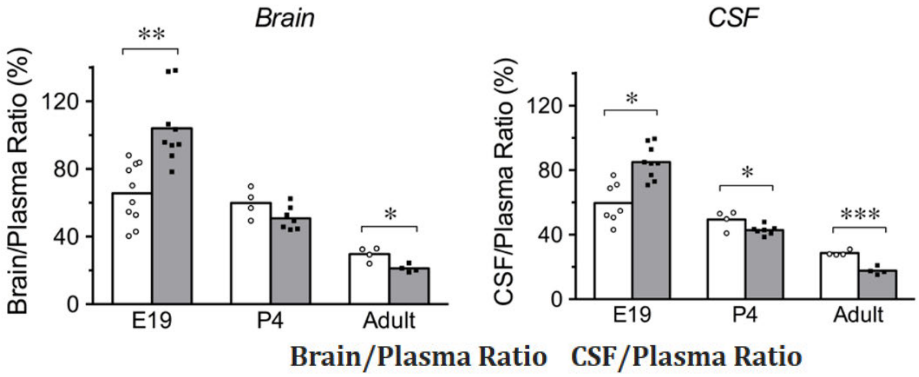
[199] Koehn et al. Determinants of drug entry into the developing brain. F1000Res. 2019 Aug 7;8:1372. doi: 10.12688/f1000research.20078.1. PMID: 31656590; PMCID: PMC6799938.

[200] Skoko et al. Loss of Nrf2 in mice evokes a congenital intrahepatic shunt that alters hepatic oxygen and protein expression gradients and toxicity. Toxicol Sci. 2014 Sep;141(1):112-9. doi: 10.1093/toxsci/kfu109. Epub 2014 Jun 12. PMID: 24924401; PMCID: PMC4271119.

(A) Brain (DPM/µg), CSF (DPM/µl) and Plasma (DPM/µl) Radioactivity Levels

		Acute				Chronic			
Paracetamol		n	Brain	CSF	Plasma	n	Brain	CSF	Plasma
E19	Mean	10	39.6	35.3	57.8	9	146.1	119.0	140.5
	SD	(7)	14.2	13.3	12.3		32.5	16.6	15.6
P4	Mean	4	117.5	96.5	193.6	7	84.4	71.0	166.8
	SD		32.4	23.1	27.6		19.2	14.5	39.4
Adult	Mean	4	27.3	25.4	90.1	4	17.6	14.9	83.3
	SD		20.1	15.8	58.7		4.2	4.5	18.5

(B) Concentration Ratios



	Brain/Plasma Ratio		CSF/Plasma Ratio	
Paracetamol	Acute	Chronic	Acute	Chronic
E19	66 ± 18	104 ± 21	60 ± 13	85 ± 10
P4	60 ± 9	51 ± 7	49 ± 6	43 ± 3
Adult	30 ± 4	21 ± 2	29 ± 2	18 ± 2

Figure 19. Brain, CSF, and Plasma Concentrations for Acetaminophen (APAP). APAP concentrations (A, left) at 30 min, (A, right) at 2.5 hours, and (B) the ratio of APAP in brain/plasma at 30minutes (left, white) and 2.5 hours (right, grey). Adults were non-pregnant females and littermates of both sexes were included in the E19 and P4 age groups of pups. SD=standard deviation. * $p<0.05$, ** $p<0.01$, two-tailed Student's t-test. DPM = disintegrations per minute. [201]

ii. Oxidative Stress and the APAP Metabolites, NAPQI and AM404, in the Brain

The harmful mechanisms described above will reach the brain of the developing fetus. NAPQI itself is generated in brain tissues. APAP easily crosses the blood brain barrier and is distributed uniformly throughout the central nervous system.[202] The liver produces the largest amount of the CYP2E1 enzyme

[201] Koehn et al. Determinants of drug entry into the developing brain. F1000Res. 2019 Aug 7;8:1372. doi: 10.12688/f1000research.20078.1. PMID: 31656590; PMCID: PMC6799938.

[202] Ghanen, C., Perez Mariz, J., Manautou, J., Mottino, A., Acetaminophen; From liver to brain: new insights into drug pharmacological action and toxicity, Pharmacol Res. 2016 July; 109: 119-131, DOI: 10.1016/ j.phrs.2016.02.020, p. 2.

that causes APAP to form the toxic metabolite NAPQI. But the brain also produces CYP2E1 (and other P450 cytochromes), including in the frontal lobe, cerebellum, occipital lobe, and pons regions.[203,204] As a result, APAP present in brain tissue can metabolize into toxic NAPQI. It has been reported that when APAP causes oxidative stress in the brain, it decreases GSH and increases oxidative stress in almost every region of the central nervous system.[205] CYP2E1 is also found in mouse (E17),[206] rat,[207] monkey brain,[208] and prenatal human brain.[209] Epigenetic changes were also reported in CYP2E1 in the placentas from the MARBLES ASD study.[210] This indicates that NAPQI can be produced in all human brain regions examined and in prenatal human brains. CYP2E1 is also found in brain capillaries[211] and is responsive to medications.

The APAP metabolite AM404 also appears in the brain. The enzyme FAAH, which produces the endocannabinoid and serotonergic signaling molecule AM404, shows expression in neurons and throughout the central nervous system.[212] FAAH is expressed in the testis, brain, and other organs (Figure 20). Thus, APAP that reaches the brain can be metabolized into AM404 by FAAH. This gene is also reported to be expressed in human placenta[213] and has been proposed to influence implantation, miscarriage,[214] and placenta cell (trophoblast) proliferation.[215] FAAH is also reported to be expressed in

[203] Farin and Omiecinski. Regiospecific expression of cytochrome P-450s and microsomal epoxide hydrolase in human brain tissue. J Toxicol Environ Health. 1993 Oct-Nov;40(2-3):317-35. doi: 10.1080/15287399309531797. PMID: 7693960.
[204] Upadhya et al. Cytochrome P4502E (CYP2E) in brain: constitutive expression, induction by ethanol and localization by fluorescence in situ hybridization. Arch Biochem Biophys. 2000 Jan 1;373(1):23-34. doi: 10.1006/abbi.1999.1477. PMID: 10620320.
[205] Micheli et al. Effect of acetaminophen on glutathione levels in several regions of the rat brain. Curr Ther Res 1993;53:730–6. https://doi.org/10.1016/S0011-393X(05)80745-3
[206] Choudhary et al. Expression patterns of mouse and human CYP orthologs (families 1-4) during development and in different adult tissues. Arch Biochem Biophys. 2005 Apr 1;436(1):50-61. doi: 10.1016/j.abb.2005.02.001. PMID: 15752708.
[207] Joshi and Tyndale. Induction and recovery time course of rat brain CYP2E1 after nicotine treatment. Drug Metab Dispos. 2006 Apr;34(4):647-52. doi: 10.1124/dmd.105.008029. Epub 2006 Jan 24. PMID: 16434548.
[208] Joshi and Tyndale. Regional and cellular distribution of CYP2E1 in monkey brain and its induction by chronic nicotine. Neuropharmacology. 2006 Apr;50(5):568-75. doi: 10.1016/j.neuropharm.2005.11.001. Epub 2005 Dec 20. PMID: 16368115.
[209] Brzezinski et al. Catalytic activity and quantitation of cytochrome P-450 2E1 in prenatal human brain. J Pharmacol Exp Ther. 1999 Jun;289(3):1648-53. PMID: 10336564.
[210] Zhu et al. Placental DNA methylation levels at CYP2E1 and IRS2 are associated with child outcome in a prospective autism study. Hum Mol Genet. 2019 Aug 15;28(16):2659-2674. doi: 10.1093/hmg/ddz084. PMID: 31009952; PMCID: PMC6687952.
[211] Sato et al. An Atlas of the Quantitative Protein Expression of Anti-Epileptic-Drug Transporters, Metabolizing Enzymes and Tight Junctions at the Blood-Brain Barrier in Epileptic Patients. Pharmaceutics. 2021 Dec 9;13(12):2122. doi: 10.3390/pharmaceutics13122122. PMID: 34959403; PMCID: PMC8708024.
[212] Thomas et al. Fatty acid amide hydrolase, the degradative enzyme for anandamide and oleamide, has selective distribution in neurons within the rat central nervous system. J Neurosci Res. 1997 Dec 15;50(6):1047-52. doi: 10.1002/(SICI)1097-4547(19971215)50:6<1047::AID-JNR16>3.0.CO;2-1. PMID: 9452020.
[213] Park et al. Identification of the CB1 cannabinoid receptor and fatty acid amide hydrolase (FAAH) in the human placenta. Placenta. 2003 Nov;24(10):990-5. doi: 10.1016/s0143-4004(03)00165-6. PMID: 14580383.
[214] Trabucco et al. Endocannabinoid system in first trimester placenta: low FAAH and high CB1 expression characterize spontaneous miscarriage. Placenta. 2009 Jun;30(6):516-22. doi: 10.1016/j.placenta.2009.03.015. Epub 2009 May 5. PMID: 19419760.
[215] Habayeb et al. Expression of the endocannabinoid system in human first trimester placenta and its role in trophoblast proliferation. Endocrinology. 2008 Oct;149(10):5052-60. doi: 10.1210/en.2007-1799. Epub 2008 Jul 3. PMID: 18599552.

the pre-implantation mouse embryos[216] and in chicken and mouse embryos before and during neurulation.[217] APAP is also reported to cause a reduction in cerebral cortical fatty acid amide FAAH.[218]

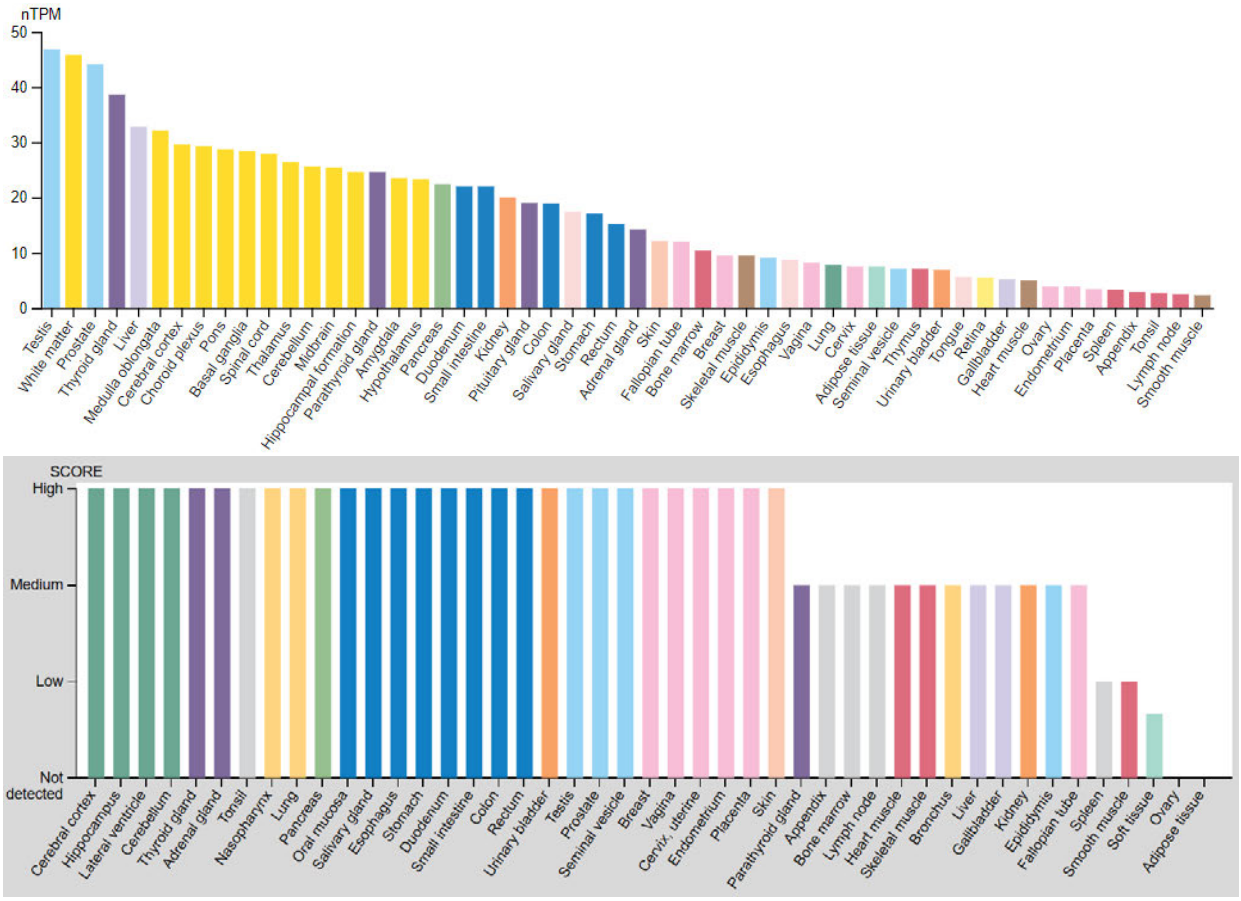


Figure 20. Gene and Protein Expression of FAAH in Humans. (Top) The consensus expression for each gene is shown by organ or tissue as normalized transcripts (nTPM). The expression of FAAH, results in the enzyme that produces AM404 from PAP, with medium transcription (>30 nTPM) in testis, brain, prostate, thyroid, and liver, and low transcription (nTPM > 2) in numerous tissues. (Bottom) Protein expression data is shown for each of the 44 tissues. Color-coding is based on 13 tissue groups, each consisting of tissues with common functional features. Level of antibody staining/expression are indicated for each tissue and are high in neural tissues.[219]

iii. Oxidative Stress and the Developing Brain

Studies in humans have demonstrated that oxidative stress is produced at "therapeutic" dosages of acetaminophen. In one study, human subjects were given dosages of .5g, 2g, or 4g spread out in four doses

[216] Paria et al. Fatty-acid amide hydrolase is expressed in the mouse uterus and embryo during the periimplantation period. Biol Reprod. 1999 May;60(5):1151-7. doi: 10.1095/biolreprod60.5.1151. PMID: 10208977.
[217] Psychoyos et al. Cannabinoid receptor 1 signaling in embryo neurodevelopment. Birth Defects Res B Dev Reprod Toxicol. 2012 Apr;95(2):137-50. doi: 10.1002/bdrb.20348. Epub 2012 Feb 6. PMID: 22311661; PMCID: PMC4175447.
[218] Philippot et al. A Cannabinoid Receptor Type 1 (CB1R) Agonist Enhances the Developmental Neurotoxicity of Acetaminophen (Paracetamol). Toxicol Sci. 2018 Nov 1;166(1):203-212. doi: 10.1093/toxsci/kfy199. PMID: 30165669.
[219] Uhlén et al. Proteomics. Tissue-based map of the human proteome. Science. 2015 Jan 23;347(6220):1260419. doi: 10.1126/science.1260419. PMID: 25613900.

over 24 hours (Figure 21).[220] The 4g dosage represents the maximum recommended human dosage over 24 hours. All of these dosages are below dosages reported to result in liver toxicity (≥ 150 mg/kg or 7.5 g in adults, see Acetaminophen Toxicity**)**. Urine and blood were sampled and genomic and metabolomic responses analyzed. The results showed that even at these "therapeutic" dosages, acetaminophen caused oxidative stress, cell death, and DNA damage.

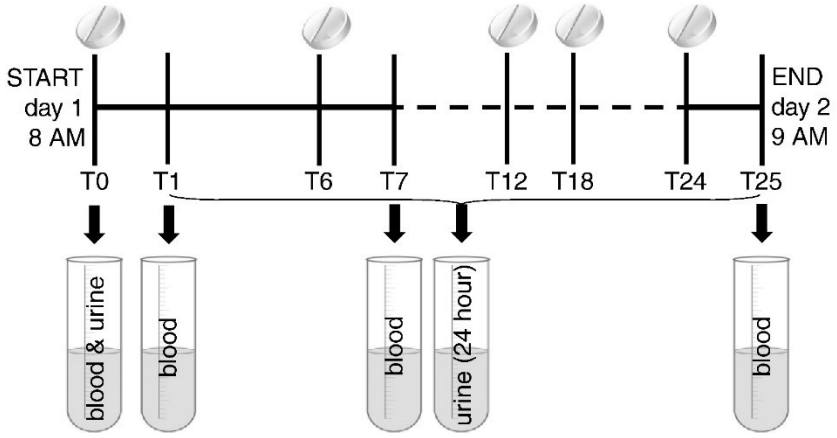


Figure 21. Sampling and Dosing Schedule. Pills indicate APAP dosing time points and tubes indicate sampling of blood and/or urine at the corresponding time point (T in hours) for each dose round. In the 4g APAP dose round, blood samples for transcriptomic analyses were not collected on T1 and T7.[221]

Following the administration of 4 grams of acetaminophen (APAP) at 25 hours (T25), there were reported signals of "apoptosis and survival/oxidative stress/DNA damage". Notably, the process most significantly affected at the 4g dose was oxidative phosphorylation, which was downregulated. Liver toxicity resulting from high and toxic doses of APAP is generally attributed to oxidative stress and impaired mitochondrial function caused by reactive metabolites, such as NAPQI, generated during APAP metabolism. This downregulation of genes involved in oxidative phosphorylation was also observed in previous studies in humans and rats.[222,223] These data support that "therapeutic" doses of APAP induce changes in gene expression consistent with an oxidative stress response. A previous clinical study used a single bolus dose of 4 grams of APAP and also reported gene expression downregulation of oxidative phosphorylation genes.[224] The authors of that study concluded that these changes resembled the gene expression patterns observed in patients who overdosed on APAP and rats receiving toxic doses. These findings indicate that even "therapeutic" doses of APAP produce oxidative stress responses in humans and animals, but this

[220] Jetten et al. 'Omics analysis of low dose acetaminophen intake demonstrates novel response pathways in humans. Toxicol Appl Pharmacol. 2012 Mar 15;259(3):320-8. doi: 10.1016/j.taap.2012.01.009. Epub 2012 Jan 20. PMID: 22285215.
[221] Jetten et al. 'Omics analysis of low dose acetaminophen intake demonstrates novel response pathways in humans. Toxicol Appl Pharmacol. 2012 Mar 15;259(3):320-8. doi: 10.1016/j.taap.2012.01.009. Epub 2012 Jan 20. PMID: 22285215.
[222] Fannin et al. Acetaminophen dosing of humans results in blood transcriptome and metabolome changes consistent with impaired oxidative phosphorylation. Hepatology. 2010 Jan;51(1):227-36. doi: 10.1002/hep.23330. PMID: 19918972; PMCID: PMC2925683.
[223] Katyare and Satav. Impaired mitochondrial oxidative energy metabolism following paracetamol-induced hepatotoxicity in the rat. Br J Pharmacol. 1989 Jan;96(1):51-8. doi: 10.1111/j.1476-5381.1989.tb11783.x. PMID: 2522334; PMCID: PMC1854327.
[224] Fannin et al. Acetaminophen dosing of humans results in blood transcriptome and metabolome changes consistent with impaired oxidative phosphorylation. Hepatology. 2010 Jan;51(1):227-36. doi: 10.1002/hep.23330. PMID: 19918972; PMCID: PMC2925683.

stress is recoverable and does not normally result in acute liver disease or severe liver injury. The authors also reasonably suggest that the liver is a resilient and regenerative organ with a large capacity for handling insults in healthy individuals, offering that the liver manages "therapeutic dose" oxidative insults without significant complications for the individual. Unfortunately, the same cannot be said for embryonic or neurodevelopmental tissues. Oxidative stress is not merely something to recover from during development, because oxidative stress alters developmental cascades of cell proliferation, differentiation, and at high levels can produce apoptosis or cell death (Figure 22).

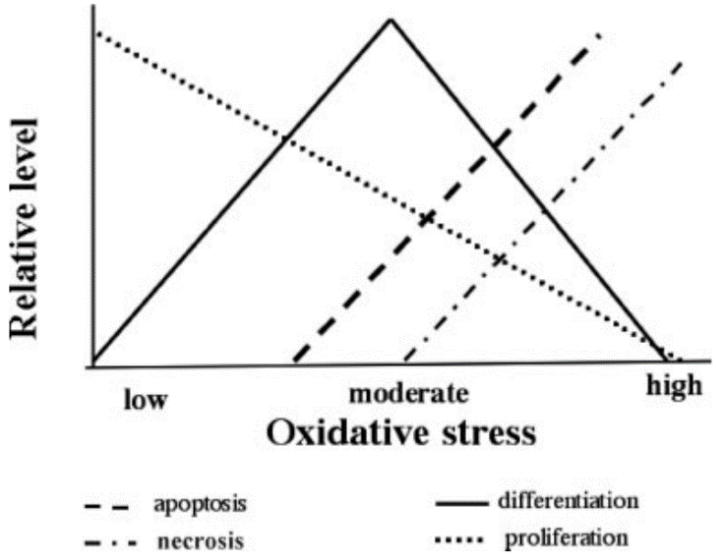


Figure 22. Effect of Reducing and Oxidative Environments on Cell Proliferation, Differentiation, Apoptosis, and Necrosis. In an environment with low oxidative stress, cells are more likely to proliferate (dotted line), whereas a moderately elevated oxidative stress will encourage differentiation (solid line). Higher oxidative stress promotes apoptosis (dashed line) and the highest oxidative environment will promote necrosis (dotted-dashed line).[225]

Multiple studies have examined the impacts of APAP on mitochondria, and these studies indicate that APAP is an inhibitor of complex III in the mitochondrial electron transport chain.[226] While these impacts (Figure 23) were initially reported at hepatotoxic dosages, a recent study reports APAP induced "loss of mitochondrial respiration was also detected below the threshold of acetaminophen toxicity."[227]

[225] Reviewed in Dennery. Effects of oxidative stress on embryonic development. Birth Defects Res C Embryo Today. 2007 Sep;81(3):155-62. doi: 10.1002/bdrc.20098. PMID: 17963268.

[226] Donnelly et al. Inhibition of mitochondrial respiration in vivo is an early event in acetaminophen-induced hepatotoxicity. Arch Toxicol. 1994;68(2):110-8. doi: 10.1007/s002040050043. PMID: 8179480; Meyers et al. Acetaminophen-induced inhibition of hepatic mitochondrial respiration in mice. Toxicol Appl Pharmacol. 1988 May;93(3):378-87. doi: 10.1016/0041-008x(88)90040-3. PMID: 3368917.

[227] Prill et al. Real-time monitoring of oxygen uptake in hepatic bioreactor shows CYP450-independent mitochondrial toxicity of acetaminophen and amiodarone. Arch Toxicol. 2016 May;90(5):1181-91. doi: 10.1007/s00204-015-1537-2. Epub 2015 Jun 4. PMID: 26041127.

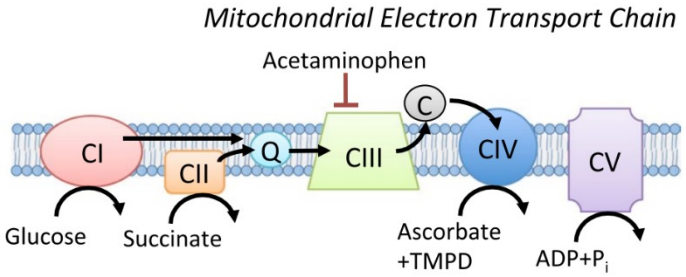


Figure 23. Acetaminophen Directly Inhibits Mitochondrial Complex III Independent of CYP450.

There are similar reports in the brain, such that NAPQI "exerts oxidative stress and depletes glutathione in the brain" even at relatively low doses, below the level that results in liver toxicity.[228] This study also reported that the developing brain has lower levels of GSH than the adult brain, which makes the developing brain less resistant to oxidative stress, and more likely to have adverse impacts by oxidative stress on the developing brain. The presence of "double bonds in membrane phospholipids makes them particularly susceptible to oxidative damage" so the brain is particularly vulnerable to oxidative stress because it has high lipid content and limited antioxidant capacity.[229] As indicated previously, oxidative stress and ROS can decrease GSH, and induce DNA oxidation, and lipid peroxidation, which can disrupt the composition of membrane phospholipids and alter neuronal function.

Furthermore, some individuals and populations are reported to have differences in glutathione and APAP metabolism, which can influence the generation of and increase vulnerability to oxidative stress during neurodevelopment.[230] For example, African-Americans oxidize acetaminophen more slowly than European-Americans, which is partially explained by reported CYP2E1 polymorphisms.[231] The resultant increased exposure to NAPQI is expected to increase risk of lowering GSH, increased DNA or lipid oxidation, protein adducts, and neurodevelopmental disorders. As expected, studies have shown that children with autism often have lower levels of GSH, and increased levels of oxidized glutathione (GSSG).[232]

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[228] Bührer et al. Paracetamol (Acetaminophen) and the Developing Brain. Int J Mol Sci. 2021 Oct 15;22(20):11156. doi: 10.3390/ijms222011156. PMID: 34681816; PMCID: PMC8540524.

[229] Tamiji and Crawford. The neurobiology of lipid metabolism in autism spectrum disorders. Neurosignals. 2010;18(2):98-112. doi: 10.1159/000323189. Epub 2011 Feb 4. PMID: 21346377.

[230] Upadhya et al. Cytochrome P4502E (CYP2E) in brain: constitutive expression, induction by ethanol and localization by fluorescence in situ hybridization. Arch Biochem Biophys. 2000 Jan 1;373(1):23-34. doi: 10.1006/abbi.1999.1477. PMID: 10620320.

[231] Court MH, Zhu Z, Masse G, Duan SX, James LP, Harmatz JS, Greenblatt DJ. Race, Gender, and Genetic Polymorphism Contribute to Variability in Acetaminophen Pharmacokinetics, Metabolism, and Protein-Adduct Concentrations in Healthy African-American and European-American Volunteers. J Pharmacol Exp Ther. 2017 Sep;362(3):431-440. doi: 10.1124/jpet.117.242107. Epub 2017 Jun 29. PMID: 28663312; PMCID: PMC5562097.

[232] James et al. Metabolic biomarkers of increased oxidative stress and impaired methylation capacity in children with autism. Am J Clin Nutr. 2004 Dec;80(6):1611-7. doi: 10.1093/ajcn/80.6.1611. PMID: 15585776; Geier et al. A prospective study of transsulfuration biomarkers in autistic disorders. Neurochem Res. 2009 Feb;34(2):386-93. doi: 10.1007/s11064-008-9782-x. Epub 2008 Jul 9. Erratum in: Neurochem Res. 2009 Feb;34(2):394. PMID: 18612812.

iv. Changes in Brain Tissue Resulting from Exposure to APAP During Neurodevelopment

Numerous animal studies have demonstrated that exposing developing brains to APAP at dosages equivalent to or less than the recommended therapeutic dosage in humans causes changes in key structures in the brain consistent with the impact of APAP and its metabolites on oxidative stress, the endocannabinoid system, and prostaglandins. The animal studies on APAP are described in greater detail below. Some key findings on biological changes in the brain are provided in summary. Briefly, exposure to acetaminophen has been shown to cause the following effects on brain tissues:

- systemic depletion of glutathione and resulting brain damage (de Silva 2012)[233]
- decreased glutathione, loss of ATP production, and enlargement and fusion of mitochondria (Ruepp 2002)[234]
- acute oxidative stress in the hippocampus (Philippot 2022)[235]
- decreased glutathione and DNA damage consistent with oxidative stress (Markovic 2013)[236]
- altered prefrontal cortex gene expression related to various molecular pathways (Baker 2023)[237]
- changes in the levels of brain derived neurotropic factor (Viberg 2014)[238]
- reduction in cerebral cortical fatty acid amide hydroxylase (FAAH), and reduction of transcript levels of hippocampal synaptophysin and tropomyosin receptor kinase B (Philippot 2018)[239]
- decrease in the number of neurons in a portion of the hypothalamus (Hay-Schmidt 2017)[240]

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[233] da Silva et al. Acute brain damage induced by acetaminophen in mice: effect of diphenyl diselenide on oxidative stress and mitochondrial dysfunction. Neurotox Res. 2012 Apr;21(3):334-44. doi: 10.1007/s12640-011-9288-1. Epub 2011 Nov 12. PMID: 22081409.

[234] Ruepp et al. Genomics and proteomics analysis of acetaminophen toxicity in mouse liver. Toxicol Sci. 2002 Jan;65(1):135-50. doi: 10.1093/toxsci/65.1.135. PMID: 11752693.

[235] Philippot et al. Paracetamol (Acetaminophen) and its Effect on the Developing Mouse Brain. Front Toxicol. 2022 Mar 22;4:867748. doi: 10.3389/ftox.2022.867748. PMID: 35391823; PMCID: PMC8981466.

[236] Markovic et al. Lipid peroxidation, detoxification capacity, and genome damage in mice after transplacental exposure to pharmaceutical drugs. Braz J Med Biol Res. 2013 Dec;46(12):1014-1020. doi: 10.1590/1414-431X20132814. Epub 2013 Dec 10. PMID: 24345909; PMCID: PMC3935272.

[237] Baker et al. Sex-specific neurobehavioral and prefrontal cortex gene expression alterations following developmental acetaminophen exposure in mice. Neurobiol Dis. 2023 Feb;177:105970. doi: 10.1016/j.nbd.2022.105970. Epub 2022 Dec 19. PMID: 36549432; PMCID: PMC9940030.

[238] Viberg et al. Paracetamol (acetaminophen) administration during neonatal brain development affects cognitive function and alters its analgesic and anxiolytic response in adult male mice. Toxicol Sci. 2014 Mar;138(1):139-47. doi: 10.1093/toxsci/kft329. Epub 2013 Dec 21. PMID: 24361869.

[239] Philippot et al. A Cannabinoid Receptor Type 1 (CB1R) Agonist Enhances the Developmental Neurotoxicity of Acetaminophen (Paracetamol). Toxicol Sci. 2018 Nov 1;166(1):203-212. doi: 10.1093/toxsci/kfy199. PMID: 30165669.

[240] Hay-Schmidt et al. Prenatal exposure to paracetamol/acetaminophen and precursor aniline impairs masculinisation of male brain and behaviour. Reproduction. 2017 Aug;154(2):145-152. doi: 10.1530/REP-17-0165. Epub 2017 May 30. PMID: 28559473.

- decreased glutathione in the brain and oxidative stress increases (Micheli 1993)[241]
- increased spinophilin in the cerebellar cortex altered growth of dendritic spines (Dean 2012)[242]
- increased oxidative stress markers in brain tissue (Saeedan 2018)[243]
- increased inflammation, IL1β (Koehn 2020)[244]
- altered levels of amino acids in brain regions including the prefrontal cortex, striatum, and hypothalamus (Blecharz-Klin 2014)[245]
- altered levels of neurotransmitters in the hypothalamus (Blecharz-Klin 2019)[246]
- decreased glutathione and increased DNA fragmentation in brain tissue, and cell death and DNA damage in brain tissue (Posadas 2010)[247]

Summary of tissue interactions

APAP readily crosses the placenta and the blood-brain barrier to reach the developing brain. Animal studies report that concentrations of APAP are two to seven times higher in the developing brain than in maternal tissues. Tissues of the brain are more susceptible to oxidative stress than organs such as the liver. These tissue and organ interactions indicate that APAP and/or metabolites of APAP, such as NAPQI, cause oxidative stress, and dose responsive neurotoxicity, and these data are consistent with oxidative stress AOP 22 (Figure 11). In addition, cannabinoid, prostaglandin, and serotonergic signaling are essential for normal tissue and organ developmental, and interactions of APAP and/or metabolites, such as PAP and AM404, with these pathways operate as a branch of this AOP.

[241] Micheli et al. Effect of acetaminophen on glutathione levels in several regions of the rat brain. Curr Ther Res 1993;53:730–6. https://doi.org/10.1016/S0011-393X(05)80745-3

[242] Dean et al. Prostaglandin E2 is an endogenous modulator of cerebellar development and complex behavior during a sensitive postnatal period. Eur J Neurosci. 2012 Apr;35(8):1218-29. doi: 10.1111/j.1460-9568.2012.08032.x. PMID: 22512254; PMCID: PMC3534986.

[243] Saeedan et al. Effect of early natal supplementation of paracetamol on attenuation of exotoxin/endotoxin induced pyrexia and precipitation of autistic like features in albino rats. Inflammopharmacology. 2018 Aug;26(4):951-961. doi: 10.1007/s10787-017-0440-2. Epub 2018 Jan 11. PMID: 29327281.

[244] Koehn et al. Effects of paracetamol (acetaminophen) on gene expression and permeability properties of the rat placenta and fetal brain. F1000Res. 2020 Jun 8;9:573. doi: 10.12688/f1000research.24119.2. PMID: 32934805; PMCID: PMC7477648.

[245] Blecharz-Klin et al. Paracetamol impairs the profile of amino acids in the rat brain. Environ Toxicol Pharmacol. 2014 Jan;37(1):95-102. doi: 10.1016/j.etap.2013.11.004. Epub 2013 Nov 13. PMID: 24316461.

[246] Blecharz-Klin et al. Hypothalamus - Response to early paracetamol exposure in male rats offspring. Int J Dev Neurosci. 2019 Aug;76:1-5. doi: 10.1016/j.ijdevneu.2019.05.004. Epub 2019 May 21. PMID: 31125683.

[247] Posadas et al. Acetaminophen induces apoptosis in rat cortical neurons. PLoS One. 2010 Dec 10;5(12):e15360. doi: 10.1371/journal.pone.0015360. PMID: 21170329; PMCID: PMC3000821.

4. Phenotype

As discussed above, APAP produces the toxic metabolite NAPQI, which can cause oxidative stress and adduct formation (the addition of NAPQI to another molecule) and detoxification is accomplished by conjugation of NAPQI with glutathione (GSH). Individuals who are more susceptible to increased NAPQI production or decreased GSH levels are expected to be more susceptible to this AOP. Several studies have demonstrated biological markers in ASD patients consistent with this oxidative stress AOP. Studies have reported that ASD patients have a deficiency in methionine and cysteine, which are precursors in the production of glutathione.[248] It has also been shown that children with autism have lower levels of the reduced form of glutathione (GSH).[249] Patients with ASD have greater markers of oxidative stress.[250]

As discussed above, acetaminophen also alters endocannabinoid signaling, which is required for proper axon growth and positioning. Consistent with this mode of action, a number of studies provide evidence of dysregulated axonal growth and guidance as key developmental processes (key events) underlying the clinical manifestations of ASD.[251] It has also been reported that subjects with ASD have decreased neuronal and interneuron expression in the prefrontal cortex and cerebellum.[252] In addition, it has been hypothesized that exposure to acetaminophen disrupts axon growth and positioning and "could result in improper development of the corpus callosum, which provides a pathway for axons traversing the brain from one hemisphere to the other."[253] Consistent with the mode of action, it has been shown that individuals with ASD have reduced axon numbers, axon diameter, and axon area in the corpus callosum.[254]

As reviewed above, the APAP metabolite AM404 increases the level and activity of the endogenous cannabinoid, anandamide, that targets the CB receptors. It has been hypothesized that repeated exposure during brain development to APAP would result in above-normal levels of anandamide, which would cause downregulation of anandamide synthesis, and thereby lower levels of anandamide in later life.[253] Consistent with this mode of action, it has been shown that children with ASD have lower levels of the

[248] James et al. Metabolic biomarkers of increased oxidative stress and impaired methylation capacity in children with autism. Am J Clin Nutr. 2004 Dec;80(6):1611-7. doi: 10.1093/ajcn/80.6.1611. PMID: 15585776; James et al. Metabolic endophenotype and related genotypes are associated with oxidative stress in children with autism. Am J Med Genet B Neuropsychiatr Genet. 2006 Dec 5;141B(8):947-56. doi: 10.1002/ajmg.b.30366. PMID: 16917939; PMCID: PMC2610366; James et al. Cellular and mitochondrial glutathione redox imbalance in lymphoblastoid cells derived from children with autism. FASEB J. 2009 Aug;23(8):2374-83. doi: 10.1096/fj.08-128926. Epub 2009 Mar 23. PMID: 19307255; PMCID: PMC2717775.

[249] Tamiji and Crawford. The neurobiology of lipid metabolism in autism spectrum disorders. Neurosignals. 2010;18(2):98-112. doi: 10.1159/000323189. Epub 2011 Feb 4. PMID: 21346377.

[250] Howsmon et al. Classification and adaptive behavior prediction of children with autism spectrum disorder based upon multivariate data analysis of markers of oxidative stress and DNA methylation. PLoS Comput Biol. 2017 Mar 16;13(3):e1005385. doi: 10.1371/journal.pcbi.1005385. PMID: 28301476; PMCID: PMC5354243.

[251] McFadden and Minshew. Evidence for dysregulation of axonal growth and guidance in the etiology of ASD. Front Hum Neurosci. 2013 Oct 22;7:671. doi: 10.3389/fnhum.2013.00671. PMID: 24155705; PMCID: PMC3804918.

[252] Edmonson et al. Altered glial marker expression in autistic post-mortem prefrontal cortex and cerebellum. Mol Autism. 2014 Jan 10;5(1):3. doi: 10.1186/2040-2392-5-3. PMID: 24410870; PMCID: PMC3914711.

[253] Schulz, S. et al., Endocannabinoid system dysregulation from acetaminophen use may lead to autism spectrum disorder: Could cannabinoid treatment be efficacious?, 2021 Molecules 26:1845, DOI: 10.3390/ molecules26071845

[254] Wegiel et al. Deficit of corpus callosum axons, reduced axon diameter and decreased area are markers of abnormal development of interhemispheric connections in autistic subjects. Acta Neuropathol Commun. 2018 Dec 19;6(1):143. doi: 10.1186/s40478-018-0645-7. PMID: 30567587; PMCID: PMC6299595.

enzyme (NAPE-PLD) that synthesizes anandamide and lower circulating levels of anandamide and other cannabinols.[255]

As discussed above, the COX and endocannabinoid system also regulate inflammation and immune signaling, particularly in the brain, and disruption by APAP is expected to produce an increased inflammatory state in the brain, which would work synergistically with the effect of NAPQI in generating oxidative stress. Consistent with that mechanism, therapeutic dosages of APAP can result in immune signaling[256] and the brains of individuals with ASD show increased levels of four main pro-inflammatory cytokines.[257]

The significant disruption of proliferation, differentiation, and neural migration and pathway development is expected to result in too many, too few, or the wrong type of cells to establish specific neural tracts and pathways. If in response to periodic periods of higher oxidative stress, the developing brain upregulates antioxidant mechanisms to lower oxidative stress, this can create a reduced oxidative stress environment in specific brain regions that increases cell proliferation and therefore more neurons. Consistent with this mode of action, several studies have shown patterns of specific areas of brain overgrowth in some individuals with ASD. For example, a postmortem study of childhood ASD found that the prefrontal cortex of ASD subjects had 67% more neurons (mean 1.94 billion; 95% CI, 1.57-2.31) compared with control children (mean 1.16 billion; 95% CI, 0.90-1.42; P = .002).[258] Other studies have shown early brain overgrowth and brain enlargement in individuals diagnosed with ASD.[259] On the other hand, when the developing brain cannot respond sufficiently to oxidative stress, increased oxidation can cause a shift from proliferation to premature differentiation, and an oxidizing environment in specific brain regions would thereby lead to disorganization of cortical layers. Consistent with this mode of action, multiple studies have reported patches of disorganization[260] and functional disruption of neuronal tracts in children with ASD.[261]

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[255] Siniscalco et al. Cannabinoid receptor type 2, but not type 1, is up-regulated in peripheral blood mononuclear cells of children affected by autistic disorders. J Autism Dev Disord. 2013 Nov;43(11):2686-95. doi: 10.1007/s10803-013-1824-9. PMID: 23585028; Aran et al. Brief Report: Cannabidiol-Rich Cannabis in Children with Autism Spectrum Disorder and Severe Behavioral Problems-A Retrospective Feasibility Study. J Autism Dev Disord. 2019 Mar;49(3):1284-1288. doi: 10.1007/s10803-018-3808-2. PMID: 30382443; De Pol and Kolla. Endocannabinoid markers in autism spectrum disorder: A scoping review of human studies. Psychiatry Res. 2021 Dec;306:114256. doi: 10.1016/j.psychres.2021.114256. Epub 2021 Oct 31. PMID: 34775294.

[256] Jetten et al. 'Omics analysis of low dose acetaminophen intake demonstrates novel response pathways in humans. Toxicol Appl Pharmacol. 2012 Mar 15;259(3):320-8. doi: 10.1016/j.taap.2012.01.009. Epub 2012 Jan 20. PMID: 22285215

[257] Brigida et al. Endocannabinod Signal Dysregulation in Autism Spectrum Disorders: A Correlation Link between Inflammatory State and Neuro-Immune Alterations. Int J Mol Sci. 2017 Jul 3;18(7):1425. doi: 10.3390/ijms18071425. PMID: 28671614; PMCID: PMC5535916.

[258] Courchesne et al. Neuron number and size in prefrontal cortex of children with autism. JAMA. 2011 Nov 9;306(18):2001-10. doi: 10.1001/jama.2011.1638. PMID: 22068992.

[259] Hazlett et al. Early brain overgrowth in autism associated with an increase in cortical surface area before age 2 years. Arch Gen Psychiatry. 2011 May;68(5):467-76. doi: 10.1001/archgenpsychiatry.2011.39. PMID: 21536976; PMCID: PMC3315057; Hazlett et al. Early brain development in infants at high risk for autism spectrum disorder. Nature. 2017 Feb 15;542(7641):348-351. doi: 10.1038/nature21369. PMID: 28202961; PMCID: PMC5336143; De Pol and Kolla. Endocannabinoid markers in autism spectrum disorder: A scoping review of human studies. Psychiatry Res. 2021 Dec;306:114256. doi: 10.1016/j.psychres.2021.114256. Epub 2021 Oct 31. PMID: 34775294.

[260] Stoner et al. Patches of disorganization in the neocortex of children with autism. N Engl J Med. 2014 Mar 27;370(13):1209-1219. doi: 10.1056/NEJMoa1307491. PMID: 24670167; PMCID: PMC4499461.

[261] Geng et al. Abnormalities of EEG Functional Connectivity and Effective Connectivity in Children with Autism Spectrum Disorder. Brain Sci. 2023 Jan 12;13(1):130. doi: 10.3390/brainsci13010130. PMID: 36672111; PMCID: PMC9857308.

The term "patches of disorganization" was used by Stoner et al. to describe the neocortical features found in the pathology of brains from children diagnosed with ASD.[260] This study systematically examined neocortical architecture in deceased children with and without ASD. The study used *in situ* hybridization with a panel of layer- and cell-type–specific molecular markers to phenotype cortical microstructure. The study assayed markers for neurons and glia, along with genes that have been implicated in the risk of autism, in prefrontal, temporal, and occipital neocortical tissue from postmortem samples obtained from children with autism and unaffected children between the ages of 2 and 15 years. The study reported focal patches of abnormal laminar cytoarchitecture and cortical disorganization of neurons, but not glia, in prefrontal and temporal cortical tissue from 10 of 11 children with autism and from 1 of 11 unaffected children. The study observed heterogeneity between cases with respect to cell types that were most abnormal in the patches and the layers that were most affected by the pathological features. Though relatively small and exploratory, the reported focal disruption of cortical laminar architecture in the cortexes of a majority of young children with autism was a novel finding.

Another research group examined functional brain connectivity as a biomarker for ASD.[261] The study enrolled a total of 179 children aged 3−10 years (90 typically developed (TD) and 89 with ASD). The study used a weighted phase lag index and a directed transfer function to investigate the functional and effective connectivity in children with ASD and TD. The study found that patients with ASD had local hyper-connectivity of brain regions in functional connectivity and simultaneous significant decrease in effective connectivity across hemispheres. The authors propose that electroencephalogram (EEG) functional connectivity abnormalities could be a potential biomarker for ASD.

A similar study compared whole-brain within-connectivity and between-connectivity between ASD individuals and TD controls in three age groups including children (< 11 years), adolescents (11-18 years), and adults (> 18 years), each comprising 21 ASD individuals and 21 TD controls.[262] The age groups were matched for age, Full IQ, and gender. Independent component analysis and dual regression were used to investigate within-connectivity. The full and partial correlations between ICs were used to investigate between-connectivity. The study found that examination of the within-connectivity showed hyper-connectivity, especially in cerebellum and brainstem in ASD children but both hyper/hypo connectivity in adolescents and ASD adults. In ASD children, differences in the between-connectivity among default mode network (DMN), salience-executive network, and fronto-parietal network were observed. There was also a negative correlation between DMN and temporal network. This study indicates that functional connectivity abnormalities in ASD may be influenced by and need to be controlled for age.

Berto et al. tested gene expression covaries with brain activity as measured by resting state functional magnetic resonance imaging (MRI) to examine if genomic differences driven by disease state can affect this relationship.[263] The study integrated data from the ABIDE I and II imaging cohorts with datasets of gene expression in brains of neurotypical individuals and individuals with ASD with regionally matched brain activity measurements from fMRI datasets. The authors reported that they identified genes linked with brain activity whose association is disrupted in ASD. They identified a subset of genes that showed

[262] Haghighat et al. Functional Networks Abnormalities in Autism Spectrum Disorder: Age-Related Hypo and Hyper Connectivity. Brain Topogr. 2021 May;34(3):306-322. doi: 10.1007/s10548-021-00831-7. Epub 2021 Apr 27. PMID: 33905003.

[263] Berto et al. Association between resting-state functional brain connectivity and gene expression is altered in autism spectrum disorder. Nat Commun. 2022 Jun 9;13(1):3328. doi: 10.1038/s41467-022-31053-5. PMID: 35680911; PMCID: PMC9184501.

a differential developmental trajectory in individuals with ASD compared with controls. These genes are enriched in voltage-gated ion channels and inhibitory neurons, pointing to excitation-inhibition imbalance in ASD. These data suggests that the association between resting-state functional brain connectivity and gene expression is altered in ASD.

A recent review on functional brain imaging in ASD reports inter-hemispheric resting state functional connectivity may be a more promising biomarker for assisting in ASD diagnosis than abnormalities in either brain-wide resting state functional connectivity or brain structure. [264] If the developing brain is exposed to excess oxidative stress, specific brain regions are expected to undergo apoptosis. Consistent with this mode of action, in individuals with ASD, some areas of the brain show decreased neural connections, termed hypo-connectivity.[265]

In short, the oxidative stress AOP (Figure 11. Oxidative Stress and Developmental Impairment of Learning and Memory) and oxidative environment mode of action (Figure 22) model the pathways and impacts of APAP observed in the brains of individuals with ASD**.**

The nutritional state of the mother can exacerbate the adverse effects of APAP on the fetus. APAP uptake from the gastrointestinal tract depends on multiple factors. APAP toxicity has been modified experimentally using normal diets versus dietary restrictions. In one such experiment, rats fed ad libitum fed (ALF) or restricted fed (RF) rats were assigned to three exposure groups (n = 8/group): 0, 300 and 500mg/kg APAP for 99 days under each feeding condition.[266] The results indicated that RF rats were more sensitive to APAP-induced hepatotoxicity than the ALF rats. This model thereby demonstrates the importance of the nutritional state to APAP-induced chronic hepatotoxicity, because nutritional status that would otherwise be unremarkable can result in hepatotoxicity when coupled withan APAP exposure. As an example of this interaction, malnourishment-associated APAP toxicity has been reported clinically during pregnancy,[267] with increased levels of 5-oxoprolinemia, which is reported to result from depletion of GSH.

It is also known that GSH is essential for embryogenesis, as knockout of glutathione synthase, the enzyme that produces GSH, results in embryo-lethality.[268] Glutathione S-transferase Pi (Gstp) was previously reported to be the predominate transferase for NAPQI coupling to GSH.[269] While humans have only one GSTP protein, there are three GSTP proteins in mice (GSTP1, GSTP2, and GSTP3). Researchers report

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[264] Yao S, Becker B, Kendrick KM. Reduced Inter-hemispheric Resting State Functional Connectivity and Its Association With Social Deficits in Autism. Front Psychiatry. 2021 Mar 4;12:629870. doi: 10.3389/fpsyt.2021.629870. PMID: 33746796; PMCID: PMC7969641.

[265] Haghighat et al. Functional Networks Abnormalities in Autism Spectrum Disorder: Age-Related Hypo and Hyper Connectivity. Brain Topogr. 2021 May;34(3):306-322. doi: 10.1007/s10548-021-00831-7. Epub 2021 Apr 27. PMID: 33905003.

[266] Kondo et al. Enhancement of acetaminophen-induced chronic hepatotoxicity in restricted fed rats: a nonclinical approach to acetaminophen-induced chronic hepatotoxicity in susceptible patients. J Toxicol Sci. 2012;37(5):911-29. doi: 10.2131/jts.37.911. PMID: 23038001.

[267] Dom et al. Malnourishment-Associated Acetaminophen Toxicity in Pregnancy. Obstet Gynecol. 2021 May 1;137(5):877-880. doi: 10.1097/AOG.0000000000004351. PMID: 33831932.

[268] Winkler et al. Glutathione is essential for early embryogenesis--analysis of a glutathione synthetase knockout mouse. Biochem Biophys Res Commun. 2011 Aug 19;412(1):121-6. doi: 10.1016/j.bbrc.2011.07.056. Epub 2011 Jul 23. PMID: 21802407.

[269] Coles et al. The spontaneous and enzymatic reaction of N-acetyl-p-benzoquinonimine with glutathione: a stopped-flow kinetic study. Arch Biochem Biophys. 1988 Jul;264(1):253-60. doi: 10.1016/0003-9861(88)90592-9. PMID: 3395122.

that Gstp1 was expressed in the cortex starting at E15.5, while Gstp2 and Gstp3 began expressing at E18.5.[270] This same report showed that knockdown of Gstp1 and Gstp2 resulted in a decrease in the number of neurites in cortical neurons, suggesting their involvement in neurite initiation. In vivo knockdown of Gstp1 and Gstp2 in layer 2/3 pyramidal neurons using in utero electroporation (IUE) showed abnormal swelling of the apical dendrite at PND3 and reduced neurite number at PND15. Chemical inhibition of GSTpi induced a significant increase in growth and increased growth hormone levels in blood and pituitary and insulin-like growth factor levels in the liver and blood of treated mice.[271] Increased head circumferences, weight, BMI, and elevated insulin-like growth factor levels are also reported in children with ASD.[272]

Clinically, glutathione synthase deficiency can result genetically due to an autosomal recessive disorder, and produces a range of phenotypes, from mild hemolytic anemia[273] and metabolic acidosis to severe neurologic disorders[274] or even death in infancy.[275] If the interaction of APAP metabolism, via NAPQI, depletes GSH, similar to loss or mutations of glutathione synthase, it is expected to result in reduction or depletion of GSH, elevated 5-oxoprolinemia, and neurologic disorders or even death in infancy. It is no coincidence that APAP exposure has also been reported to produce similar clinical presentations. In one such example, chronic APAP exposure, combined with low caloric intake and weight loss, was associated with excess 5-oxoproline and metabolic acidosis.[276] The excess 5-oxoproline is proposed to be caused by depleted GSH (Figure 24). Moreover, NAPQI has also been reported to inhibit glutathione synthetase, consistent with this mechanism.[277]

[270] Liu et al. Glutathione S-transferase Pi (Gstp) proteins regulate neuritogenesis in the developing cerebral cortex. Hum Mol Genet. 2021 Mar 25;30(1):30-45. doi: 10.1093/hmg/ddab003. PMID: 33437989; PMCID: PMC8033146.

[271] Dong et al. Glutathione S-Transferase Pi is Involved in the Growth of Mice. Chin J Physiol. 2018 Aug 31;61(4):230-239. doi: 10.4077/CJP.2018.BAG527. PMID: 30139245.

[272] Mills et al. Elevated levels of growth-related hormones in autism and autism spectrum disorder. Clin Endocrinol (Oxf). 2007 Aug;67(2):230-7. doi: 10.1111/j.1365-2265.2007.02868.x. Epub 2007 Jun 4. PMID: 17547689.

[273] Signolet et al. Recurrent Isolated Neonatal Hemolytic Anemia: Think About Glutathione Synthetase Deficiency. Pediatrics. 2016 Sep;138(3):e20154324. doi: 10.1542/peds.2015-4324. PMID: 27581854.

[274] Ekuni et al. Severe Hemolytic Anemia and Metabolic Acidosis at Birth with Glutathione Synthetase Deficiency and Progressive Neurological Symptoms on Follow-Up. Am J Case Rep. 2023 Apr 13;24:e938396. doi: 10.12659/AJCR.938396. PMID: 37050856; PMCID: PMC10106969.

[275] Njålsson et al. Genotype, enzyme activity, glutathione level, and clinical phenotype in patients with glutathione synthetase deficiency. Hum Genet. 2005 Apr;116(5):384-9. doi: 10.1007/s00439-005-1255-6. Epub 2005 Feb 17. PMID: 15717202.

[276] Armenian et al. 5-oxoprolinemia causing elevated anion gap metabolic acidosis in the setting of acetaminophen use. J Emerg Med. 2012 Jul;43(1):54-7. doi: 10.1016/j.jemermed.2011.06.017. Epub 2011 Oct 5. PMID: 21978879.

[277] Walker et al. The acetaminophen metabolite N-acetyl-p-benzoquinone imine (NAPQI) inhibits glutathione synthetase in vitro; a clue to the mechanism of 5-oxoprolinuric acidosis? Xenobiotica. 2017 Feb;47(2):164-175. doi: 10.3109/00498254.2016.1166533. Epub 2016 Apr 18. PMID: 27086508.

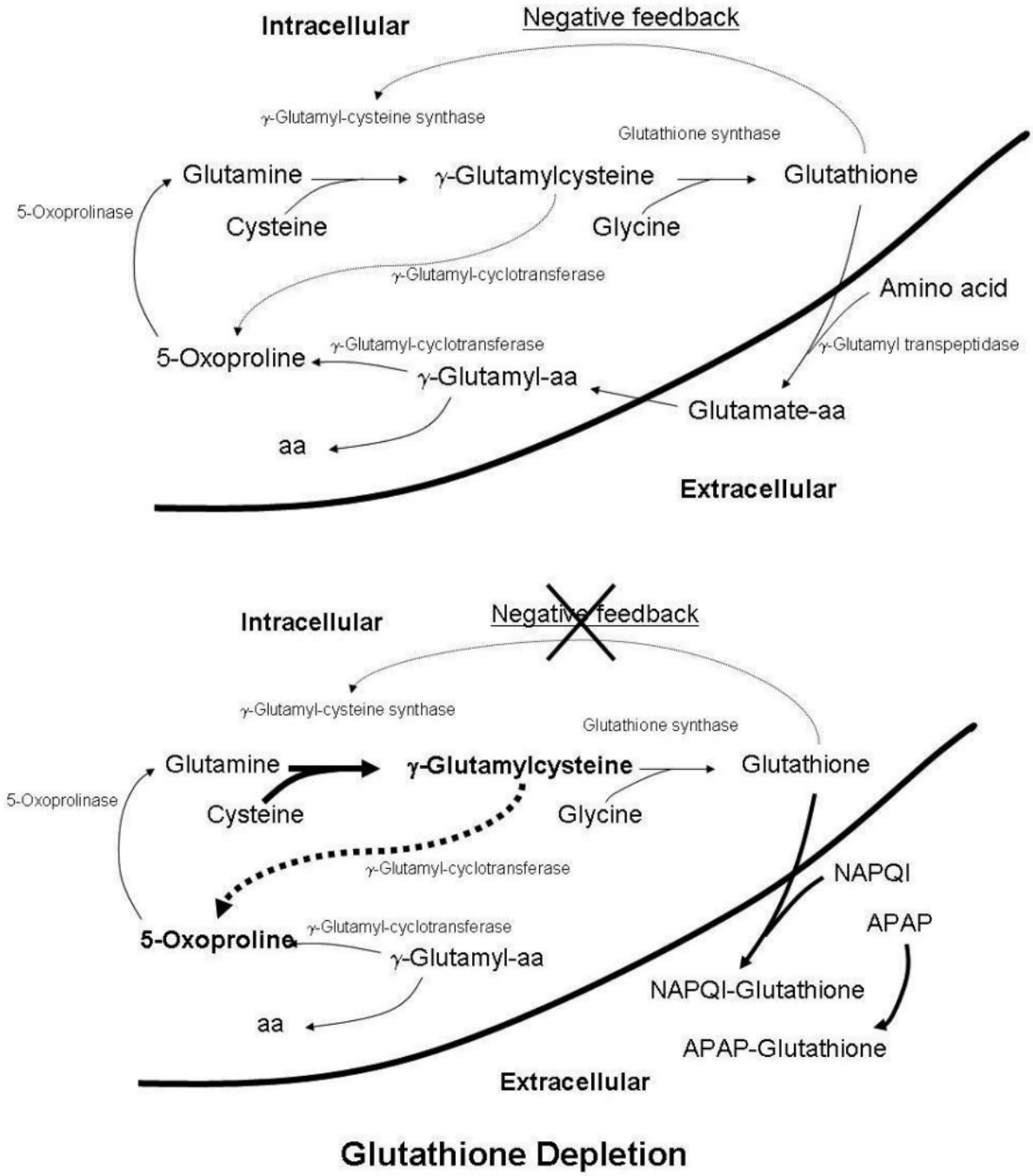

Figure 24. Interaction between Acetaminophen (APAP), NAPQI, and Glutathione. (Top) Normal function of GSH in the gamma-glutamyl pathway is diagramed, with GSH providing normal feedback inhibition of gamma-glutamyl-cysteine synthase. (Bottom) The gamma-glutamyl pathway after GSH depletion due to APAP overdose is diagramed. APAP and NAPQI deplete GSH, loss of gamma-glutamyl-cysteine synthase GSH leads to increased gamma-glutamylcysteine. This excess is converted to 5-oxoproline, which leads to 5-oxoprolinuria. Abbreviations: aa, amino acid; APAP, acetyl-para-aminophenol; NAPQI, N-acetyl-p-benzoquinone imine.[276]

These expected impacts of APAP and NAPQI on GSH interactions in various tissues can also be determined by the biodistribution of APAP. Increased levels of APAP are reported in tracer studies in the lung, liver, kidneys, pancreas, blood, stomach, and specific regions of the brain.[278] These brain regions include the frontal cortex initially (after 15 minutes), with later increases in the hippocampus (60 minutes), and cerebellum (120 minutes). As indicated below, regions of the brain most often associated with ASD, also include the frontal cortex and cerebellum. In addition to NAPQI-GSH interactions, NAPQI is also reported to interact with numerous other proteins, including albumin (Cys34).[279] While these interactions are often assumed to only be related to overdosage of APAP, NAPQI is present as a detectable metabolite at any dose, as indicated above, and clinical estimated molar dose fractions of NAPQI are higher in pregnant women than non-pregnant women, with the highest levels in the first trimester of pregnancy (median (IQR): 11% (9.1–13.4%), followed by the second (9.0% (7.5–11%)) and third (8.2% (6.8–10.1)) trimester, compared with non-pregnant women (7.7% (6.4–9.4%)).[280]

i. APAP DNA Damage and Genotoxicity in Rats

Regarding the potential impact of APAP on DNA damage, the NTP reports,[281]

> Acetaminophen and its structural analogues and metabolites indicate that this chemical is not an effective mutagen in bacterial systems but does have potential for genotoxic activity in mammalian systems by virtue of its ability to induce gene mutations, chromosomal aberrations, and unscheduled DNA synthesis.

The NTP also reviewed previous carcinogenicity as an endpoint and reported APAP,

> …is not carcinogenic when administered alone, even in the presence of severe hepatotoxicity. (NTP TR394)

At the conclusion of the NTP study[281] (NTP TR 394, p62) the report indicates that the initial finding on APAP was "some evidence of carcinogenic activity" and this was later revised by the Board of Scientific Counselors Peer Review Panel vote, so the conclusion reads,

> Under the conditions of these 2-year feed studies, there was no evidence of carcinogenic activity of acetaminophen in male F344/N rats that received 600, 3,000 or 6,000 ppm. There was equivocal evidence of carcinogenic activity of acetaminophen in female F344/N rats based on increased incidences of mononuclear cell leukemia. There was no evidence of carcinogenic activity of

[278] Lambrecht et al. Labeling of acetaminophen with I-131 and biodistribution in rats. Chem Pharm Bull (Tokyo). 2006 Feb;54(2):245-7. doi: 10.1248/cpb.54.245. PMID: 16462075.
[279] LeBlanc et al. Absolute quantitation of NAPQI-modified rat serum albumin by LC-MS/MS: monitoring acetaminophen covalent binding in vivo. Chem Res Toxicol. 2014 Sep 15;27(9):1632-9. doi: 10.1021/tx500284g. Epub 2014 Sep 5. PMID: 25168029.
[280] Brookhuis et al. Modelling Tools to Characterize Acetaminophen Pharmacokinetics in the Pregnant Population. Pharmaceutics. 2021 Aug 20;13(8):1302. doi: 10.3390/pharmaceutics13081302. PMID: 34452263; PMCID: PMC8400310.
[281] National Toxicology Program. NTP Toxicology and Carcinogenesis Studies of Acetaminophen (CAS No. 103-90-2) in F344 Rats and B6C3F1 Mice (Feed Studies). Natl Toxicol Program Tech Rep Ser. 1993 Jan;394:1-274. PMID: 12637965.

> acetaminophen in male and female B6C3F1 mice that received 600, 3,000, or 6,000 ppm. (NTP TR 394, p66)

There was also an increased incidence of mononuclear cell leukemia with APAP exposure, at all dosages tested (600-6000PPM) in rats. Mononuclear cell leukemia is not typical for this strain of rat, and this type of leukemia is unique to the rat,[282] so the risk for humans was unclear or equivocal based on these studies. These finding resulted in the conclusion of equivocal evidence of carcinogenic activity for APAP, defined as,

> Equivocal evidence of carcinogenic activity describes studies that are interpreted as showing a marginal increase of neoplasms that may be chemically related. (NTP TR 394)

ii. APAP DNA Damage and Genotoxicity in Humans

These cellular interactions indicate that APAP or metabolites of APAP, such as PAP and NAPQI, cause oxidative stress, dose responsive cytotoxicity, and DNA damage. These data are consistent with the oxidative stress AOP (See Figure 11. Oxidative Stress and Developmental Impairment of Learning and Memory). While these *in vitro* and animal studies demonstrated a potential risk in the 1990's, similar risks have now been reported in human studies. Briefly, Ross et al.[283] reported on 670 newly diagnosed myeloid leukemia cases and 701 controls aged 20 to 79 years. Results indicated regular/extra strength aspirin use was inversely associated with myeloid leukemia in women (OR = 0.59, 95% CI = 0.37-0.93) but not in men (OR = 0.85, 95% CI = 0.58-1.24). In contrast, APAP use was associated with an increased risk of myeloid leukemia in women only (OR = 1.60, 95% CI = 1.04-2.47). As aspirin has similar indications to APAP, confounding by indication is not supported in this study. In a cohort study, Walter et al.[284] examined use of APAP in relation to the incidence hematologic malignancies associated with high use (≥ 4 days/week for ≥ 4 years) of APAP (hazard ratio (HR), 1.84; 95% CI, 1.35 to 2.50 for high use; P trend = .004). This association was seen for myeloid neoplasms (HR, 2.26; 95% CI, 1.24 to 4.12), non-Hodgkin's lymphomas (HR, 1.81; 95% CI, 1.12 to 2.93), and plasma cell disorders (HR, 2.42; 95% CI, 1.08 to 5.41), but not chronic lymphocytic leukemia/small lymphocytic lymphoma (HR, 0.84; 95% CI, 0.31 to 2.28). This study does not support confounding by indication, as there was no association with risk of hematologic malignancies for increasing use of aspirin or ibuprofen. In a separate report,[285] using a hospital-based case–control study of acute leukemia, the OR associated with prior regular APAP use was 1.5 (95% CI 1.0–2.3). In summary, animal models and human studies show APAP increases leukemia risk and is specifically associated with an estimated two-fold increased risk (point estimate range: 1.5-2.3) of myeloid leukemias in humans. These animal models and human studies support that APAP or metabolites

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[282] Caldwell. Review of mononuclear cell leukemia in F-344 rat bioassays and its significance to human cancer risk: A case study using alkyl phthalates. Regul Toxicol Pharmacol. 1999 Aug;30(1):45-53. doi: 10.1006/rtph.1999.1305. PMID: 10464046.

[283] Ross et al. Nonsteroidal anti-inflammatory drug and acetaminophen use and risk of adult myeloid leukemia. Cancer Epidemiol Biomarkers Prev. 2011 Aug;20(8):1741-50. doi: 10.1158/1055-9965.EPI-11-0411. Epub 2011 Jun 29. PMID: 21715605; PMCID: PMC3153558.

[284] Walter et al. Long-term use of acetaminophen, aspirin, and other nonsteroidal anti-inflammatory drugs and risk of hematologic malignancies: results from the prospective Vitamins and Lifestyle (VITAL) study. J Clin Oncol. 2011 Jun 10;29(17):2424-31. doi: 10.1200/JCO.2011.34.6346. Epub 2011 May 9. PMID: 21555699; PMCID: PMC3107756.

[285] Weiss et al. Opposing effects of aspirin and acetaminophen use on risk of adult acute leukemia. Leuk Res. 2006 Feb;30(2):164-9. doi: 10.1016/j.leukres.2005.06.023. Epub 2005 Aug 11. PMID: 16099041.

of APAP, cause oxidative stress, DNA damage, and duration-frequency-responsive myeloid leukemia risk in humans.

A report from NIH/NICHD also found an association between APAP urinary levels and sperm DNA-fragmentation in men.[286] The study was based on males recruited for participation in the LIFE Study, a prospective cohort of 501 reproductive aged couples from 16 counties in Michigan and Texas between 2005 and 2009. This study reported that APAP and the metabolite p-aminophenol (PAP) were associated with altered quality in the sperm from men who were trying to have a baby with their partners. The researchers analyzed urine samples from the 501 men and found that higher levels of APAP in the urine were linked to lower sperm volume, weaker sperm motility, and abnormalities in the shape of sperm heads. They also found a significant increase in % DNA fragmentation of sperm in men with high urinary APAP. This damage is consistent with oxidative DNA damage by NAPQI. The expression of CYP2E1 is also observed in the male reproductive tract and sperm from mice (Figure 25).

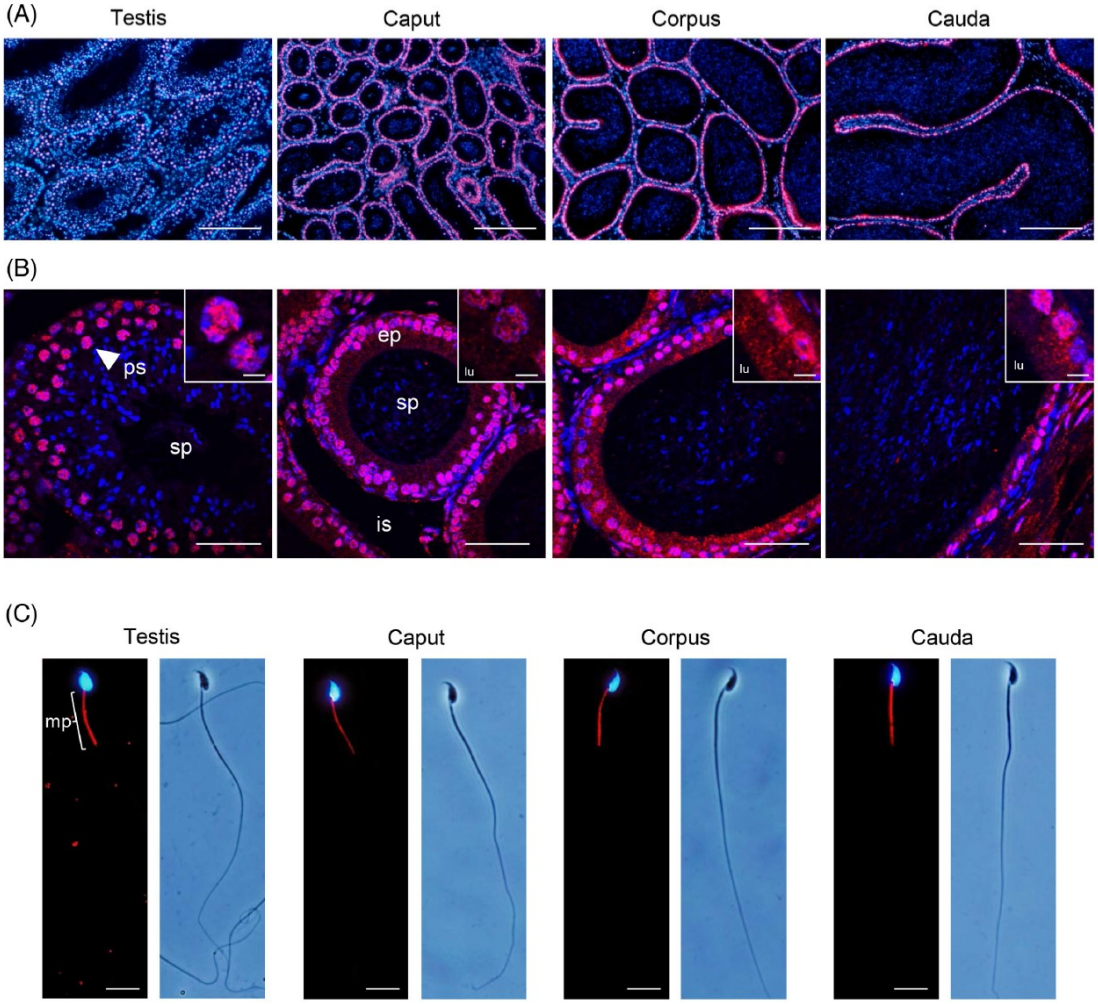

Figure 25. CYP2E1 Immunofluorescence in the Male Mouse Reproductive Tract. CYP2E1 shown in red, DAPI (DNA) shown in blue. (A) Scale bar = 200 µm. (B) Confocal images scale bar = 50 µm, inset images demonstrate higher resolution of pachytene spermatocytes (ps), and epididymal epithelium (ep). Inset scale bars are equal to 5

[286] Smarr et al. Male urinary paracetamol and semen quality. Andrology. 2017 Nov;5(6):1082-1088. doi: 10.1111/andr.12413. Epub 2017 Aug 29. PMID: 28853221.

μm. Spermatozoa (sp), the interstitial space (is) and the lumen (lu) are labeled. (C) Spermatozoa from the testis, caput, corpus, and cauda, with CYP2E1 labeling the midpiece (mp) scale bar = 25 μm.[287]

A. Animal Studies as Evidence of Causation

When assessing whether a drug exposure causes an adverse outcome, animal studies can provide important evidence that cannot be obtained from human subjects. While it would be useful to conduct carefully controlled studies using human subjects, when a drug is suspected of causing a serious adverse outcome, it would be unethical to knowingly expose humans to that serious risk of harm under the guise of an experiment. In such cases where human subjects cannot be used, animal studies can provide data from controlled experiments.

In a well-designed experiment, one group of animals, such as mice (the experimental subjects) are exposed to a drug or chemical, while another group of mice (the controls) are not. Then the two groups are subjected to identical testing, for example behavioral testing. If the test results produce different outcomes in the subject than the controls, then we can reliably conclude that the drug or chemical exposure caused the different outcome in the subjects, because the only difference between the two groups was exposure to the drug or chemical.

The physiological systems of mammals depend on common and conserved genetic, cellular, and tissue processes that have been shared and refined over time. Thus, the fundamental biology of how cells behave is shared among closely related species, allowing us to predict from animal studies what is expected to occur in humans.[288] Appropriately designed studies on rodents (mice and rats) can provide reliable results that translate to humans. Rodent biology is similar, and in many ways identical, to human biology. For example, it is estimated that 90% of mouse and human genomes show conserved synteny, or regions of with common genetic organization. The totality of genes is also similar, with an estimated 1 in 400 divergence, such that only 1 out of 400 genes studied does not have a homolog in both species. As a result, well-designed rodent studies are recognized by the FDA and scientific community as providing reliable evidence on the issue of whether a chemical exposure causes an adverse outcome in humans.

Rodent studies are particularly useful for testing the effects of chemicals or medications on neurodevelopment because rodents have genetic, biological, and behavioral characteristics that parallel those in humans. Many symptoms, pathologies, and behaviors associated with human diseases or neurological conditions can be replicated in mice and rats. However, it is important to note that while animal studies are the best available proxy for humans, they do not always translate or have the same response as humans. This is one reason why reproductive and developmental toxicity testing is performed on a rodent and non-rodent species. When results are found in multiple species, it strengthens the likelihood of risk or similar finding in humans. Regarding APAP testing specifically, both humans and rodents metabolize APAP using homologous enzymes that result in the same metabolites being produced, including NAPQI. Humans and rodents are also mammals with similar physiology and neurological development, including gastrulation, neurulation, neural tube closure, neural crest and neuronal migration, and both develop common physiological systems and neurological pathways. Humans and rodents undergo rapid brain development *in utero* and after birth, and while humans are different regarding cortical development for higher executive functions, there is shared underlying architecture in all regions of the

[287] Katen et al. Epididymal CYP2E1 plays a critical role in acrylamide-induced DNA damage in spermatozoa and paternally mediated embryonic resorptions. Biol Reprod. 2017 Apr 1;96(4):921-935. doi: 10.1093/biolre/iox021. PMID: 28379345.

[288] Daston. Laboratory models and their role in assessing teratogenesis. Am J Med Genet C Semin Med Genet. 2011 Aug 15;157C(3):183-7. doi: 10.1002/ajmg.c.30312. Epub 2011 Jul 15. PMID: 21766439.

brain. This includes major regions of the brain and patterning or neural and cortical organization and layering. Both are also susceptible to cellular and DNA damage from superoxides or free radicals, such as NAPQI, and both produce glutathione as the main antioxidant in the brain and body. We also share common neurotransmitters, including serotonergic and endocannabinoid systems that both affect fetal brain development. We also share common growth factors and neurotrophic factors, such as brain-derived neurotropic factor (BDNF) and nerve growth factor (NGF), which are important in brain development including neurogenesis and myelination, respectively.

There are, however, differences between rodents and humans that need to be accounted for in the testing. Rodents develop more rapidly than humans. For example, the period of gestation for mice is 18-20 days, rather than 9 months as in humans. Rodents are smaller with faster metabolism, which must be accounted for so that dosing of rodents can be compared to humans. For example, the accepted dose conversion for mice is that the human equivalent dose (HED) for mice is 12.3 times the human dose, adjusted for body weight. Therefore, a single 1000 mg dose of APAP (the recommended therapeutic dose for "extra strength" products) in a 60 Kg human would be 16.7 mg/Kg of body weight. For mice the HED would be 12.3 times this amount, or 205 mg/Kg of bodyweight. Similarly, a daily dose of APAP of 3,000 mg or 4,000 mg for a 60 Kg human would be 50 mg/Kg and 66.7 mg/Kg, respectively. For mice, this translates to a daily dose of 615 mg/Kg to 820 mg/Kg.

Testing for ASD and ADHD Behavior in Rodents

Both OECD and FDA guidelines recommend that behavioral testing should be conducted in two species of animals, one of which should be a non-rodent species. The guidelines also recommend that behavioral tests should be conducted using a battery of tests that assess different aspects of behavior, such as motor activity, learning and memory, anxiety, and depression.

ASD and ADHD are accompanied by certain behavioral traits. ADHD is characterized by symptoms of inattention, hyperactivity, and impulsivity. Symptoms of ASD include learning deficits, restricted or repetitive behaviors, and impaired social interactions. Since the diagnosis of ASD is currently based on behavioral criteria, modeling ASD-relevant behaviors in animals is critical for studying etiology and evaluating chemicals for risk of ASD-like outcomes. Animal models make it possible to identify biological metrics (e.g., neuroanatomy, epigenetics, microbiota, physiology) correlated with ASD-like behavior, which can shed light on the etiology of ASD, risk factors for ASD, as well as potential therapeutic targets. ASD includes learning deficits, repetitive movement, impaired social interactions, so rodent testing has been developed to ascertain similar behaviors in animals. Rodents can exhibit corresponding behaviors, including impaired social interactions, learning deficits, repetitive behaviors, and hyperactivity.

Social-emotional testing on rodents can be performed by determining whether subjects exhibit greater social caution, rearing behavior, or diminished interactions with others. One commonly used method to assess social rank among rodents is the analysis of agonistic behaviors. In the social interaction test, social and exploratory behaviors of two rodents are monitored in a familiar or unfamiliar context with different lighting conditions. The amount of time an animal spends interacting under the four test conditions is used as a measure of anxiety-like behavior. Rats and mice also show robust emotional sharing or contagion by aligning fear and pain to that of others. Brain regions necessary for emotional contagion in rodents closely resemble those associated with human empathy; understanding the biology of emotional contagion in rodents can thus shed light on human empathy.

The marble burying test is a commonly used method to assess repetitive behavior in rodents. When rodents are put in a cage with marbles, they will bury the marbles. This behavior was initially seen as anxiety

related or obsessive-compulsive disorder (OCD) behavior. When the rodents are injected with drugs used to treat anxiety or OCD, the number of marbles buried decreases. More recently, this behavior has been moved towards OCD and away from anxiety, so that marble burying and nestlet shredding tests are proposed to provide accurate and sensitive assays of repetitive and compulsive-like behaviors in rodents. This test is thereby a tool for considering OCD, ASD, and ADHD.

There are several tests that can be used to measure ADHD in rodents. One common test is the Spontaneous Alternation Test. This test measures the ability of rodents to alternate between two arms of a T-maze. Another test is the 5-Choice Serial Reaction Time Task. This test measures the ability of rodents to respond to a visual stimulus. The Continuous Performance Test is another test that can be used to measure ADHD in rodents. This test measures the ability of rodents to sustain attention over time. Tracking can be used to measure ADHD in rodents as well. For example, video tracking can be used to measure the activity levels of rodents.

Spatial learning and memory of laboratory rodents is often assessed via navigational ability in mazes, the most popular of which are the water (Morris) and dry-land (Barnes) mazes. The Morris water maze was first developed and reported by Richard G. Morris to test the spatial learning behavior of rats. The test relies on distal cues to navigate from start locations around the perimeter of an open swimming arena to locate a submerged escape platform. Spatial learning is assessed across repeated trials and reference memory is determined by testing retention of the platform location after a delay. Improved performance over sessions or trials is thought to reflect learning and memory of the escape cage/platform location. Another test is the T-maze, which examines memory and spatial learning in rodents by applying various stimuli and recording activity or responses.

Biological Testing in Rodents

Exposure to neurodevelopmental toxins can cause molecular changes and gross changes in cells or tissues, or epigenetic changes. Epigenetic mechanisms, including DNA methylation, histone modifications and non-coding RNA, are a proposed link between environmental stressors, alterations in gene expression, and phenotypes. Cytokines, growth factors, alterations in hormonal levels as well as release of stress-response and neurotropic factors are some examples of molecules that are modulated by the environment, and which come under the category of epigenome modifiers. Following cell immortalization, stressors such as environmental toxicants can induce malignant transformation.

Drawing Conclusions from Animal Studies

If a well-designed animal study demonstrates a significant adverse outcome from a drug exposure, that is a strong enough signal to prevent that drug from going forward in human clinical trials, because of the increased likelihood that the same adverse outcome would be present in humans. While some toxicities can be species specific, when different species across multiple studies reach the same conclusion, there is a high confidence that the drug causes the adverse outcome. The weight of evidence is an approach to determine the predictive power of an independent variable in relation to the dependent variable. While it would be incorrect to simply tally positive and negative findings, the totality of evidence should be considered and weight applied based on the reliability of studies and repeatability of findings.

B. Developmental Milestone in Rodents Compared to Humans

Considerable research has also been performed to examine stages of embryonic development, neurodevelopment, and comparative neurodevelopmental milestones. This topic is germane to APAP

exposures, because several rodent studies include gestational exposures, *e.g.*, single gestational day treatments (GD12.5),[289] while others performed post-natal exposures, *e.g.,* single day treatments on PND 3, 10, and 19.[290] While *in utero* exposures are generally understood to be based on common-conserved stages of organ development prior to birth, the human brain takes comparatively longer to develop both prenatally and postnatally. These latter differences in the human brain make post-natal rodent brain development more comparable to late-gestational human development. Specific examples relevant to the reviewed studies include PND3 in a rodent being comparable to ~32 weeks of human gestation, and PND10 in a rodent being ~40weeks or near term for comparable human development (Table 5). For example, Philippot et al reports that APAP exposure on PND 3 or PND 10 has neurodevelopmental impacts, but this is absent at PND19 in mice. This animal data supports a comparable risk of prenatal *in utero* (32-40 weeks of gestation) exposures for humans, but this risk is expected to diminish postnatally based on common-conserved developmental milestone.

[289] Thiele et al. Prenatal acetaminophen affects maternal immune and endocrine adaptation to pregnancy, induces placental damage, and impairs fetal development in mice. Am J Pathol. 2015 Oct;185(10):2805-18. doi: 10.1016/j.ajpath.2015.06.019. Epub 2015 Aug 5. PMID: 26254283.

[290] Philippot et al. Adult neurobehavioral alterations in male and female mice following developmental exposure to paracetamol (acetaminophen): characterization of a critical period. J Appl Toxicol. 2017 Oct;37(10):1174-1181. doi: 10.1002/jat.3473. Epub 2017 Apr 27. PMID: 28448685.

Human	Rodent	Developmental milestones	Reference (s)
23–32 wk gestation (pre-term infant)	pnd 1–3	Oligodendrocyte maturation state changes—predominance of mitotically active pre-OLs[a].	Craig et al. (2003), Lodygensky et al. (2010), Dean et al. (2011a,b)
		Immune system development.	Holsapple et al. (2003)
		Establishment of the blood-brain barrier.	Engelhardt (2003), Daneman et al. (2010)
36–40 wk gestation (term infant)	pnd 7–10	Peak brain growth spurt.	Dobbing and Sands (1979), Bockhorst et al. (2008)
		Peak in gliogenesis.	Catalani et al. (2002), Kriegstein and Alvarez-Buylla (2009)
		Increasing axonal and dendritic density.	Cowan (1979), Bockhorst et al. (2008), Baloch et al. (2009)
		Oligodendrocyte maturation state changes–switch to a pre-dominance of immature OLs.	Craig et al. (2003), Dean et al. (2011a,b)
		Consolidation of the immune system.	Holsapple et al. (2003)
2–3 year old	pnd 20–21	Brain reaches 90–95% of adult weight.	Dobbing and Sands (1973, 1979), Dekaban et al. (1987), Giedd et al. (1999)
		Peak in synaptic density at 50%> adult levels.	Huttenlocher (1979), Micheva and Beaulieu (1996)
		Peak in myelination rate.	Keshavan et al. (2002)
		Neurotransmitter and receptor changes.	Hedner et al. (1986), Romijn et al. (1991)
4–11 year old	pnd 25–35	Fractionation/specialization of prefrontal cortex neural networks (structural maturation).	Tsujimoto (2008)
		Maximum volume of grey matter and cortical thickness.	Sowell et al. (1999), Bansal et al. (2008)
12–18 year old	pnd 35–49	Reduced synapse density, reaching a plateau at adult levels.	Huttenlocher (1979), Lidow et al. (1991)
		Refinement of cognitive-dependent circuitry. Ongoing myelination; increasing white matter volume and fractional anisotrophy.	Giedd et al. (1999), Chahboune et al. (2007), Baloch et al. (2009), Brouwer et al. (2012)
20 years +	pnd 60+	Adult levels of neurotransmitters.	Romijn et al. (1991)
		Adult levels of synaptic density.	Huttenlocher (1979)
		Ongoing myelination and declining grey matter.	Lebel and Beaulieu (2011), Lebel et al. (2012)

Table 5. Summary of key developmental processes across comparable ages in humans and rodents.[291]

C. Mouse Studies on APAP: Causality and Neurodevelopmental Toxicity

I reviewed mouse studies examining whether APAP causes behavioral and biological changes consistent with ASD and ADHD, presented below. Multiple studies demonstrate that when mice are exposed to APAP at doses equivalent to human therapeutic doses *in utero* and in periods corresponding to human neurodevelopment, then APAP causes behavioral and biological changes consistent with ASD and ADHD. The behavioral changes caused by APAP exposure include social-emotional impairment, learning disabilities, hyperactivity, and repetitive behaviors. The biological changes caused by APAP exposure include reproductive toxicity, developmental toxicity, and neurotoxicity. Mice studies have demonstrated

[291] Semple et al. Brain development in rodents and humans: Identifying benchmarks of maturation and vulnerability to injury across species. Prog Neurobiol. 2013 Jul-Aug;106-107:1-16. doi: 10.1016/j.pneurobio.2013.04.001. Epub 2013 Apr 11. PMID: 23583307; PMCID: PMC3737272.

these effects from APAP doses corresponding to a human equivalent dose (in a 60Kg human) in the range of 500 mg to 732 mg per day for 1-4 days, and even as low as a single exposure of 292 mg. For comparison, the single tablet regular strength dose for humans is 325 mg, the full extra-strength dose is 1000 mg, and the daily dose is 3000 to 4000 mg.

The following mouse studies were reviewed with regard to general, reproductive, developmental, or neurodevelopmental toxicity of APAP in mice (Table 6). The strategy for identifying relevant studies is described in Appendix B.

Author	Year	Strain	Dose (mg/Kg)
Nebert et al.	1976	AKR	0, 250, 500, 1000
Alhava et al.	1978	NMRI	0, 500, 700, or 850 in 50% propylene glycol
Mancini et al.	1980	CD-1	800 - 3850
Larrey et al.	1986	CD-1	100, 200, 300, 400
Ferguson et al.	1990	B6xC3 F1	0, 100, 200, 300, 400 (IP) 500 (gastric)
Adamson et al.	1991	Swiss	0, 300, 500
Reel et al.	1992	CD-1	0, 0.25%, 0.5%, 1% = 0, 375, 715, 1430mg/Kg
NTP	1993	B6xC3 F1	0, 250, 500, 1000, 2000, 4000 PPM = 0.025%-0.4 % 0, 800,1600, 3200, 6200, 12500, 25000 PPM = 0.08-2.5 % 0, 600,3000, 6000 PPM = 0.06-0.6 %
Patel et al.	2001	C57BL/6	0, 100, 200, 250
Ruepp et al.	2002	CD-1	0, 150, 500
Ishida et al.	2007	ddY	0, 15.1, 69.5, 302.3
Leroux et al.	2010	NMRI	0, 10ug, 100ug in 10uL, not weight adjusted
Stamper et al.	2011	C57BL/6	0, 300
Gould et al.	2012	BTBR, C57, 129	0, 1, 10, 100, 400
Markovic et al.	2013	BALB/c	0, 60
Viberg et al.	2014	NMRI	0, 30 (at 0), repeated 30 (at 0 and 4hr)
Holm et al.	2015	C57BL/6J	0, 50
Lee et al.	2015	BALB/c	0, ~5
Thiele et al	2015	C57BL/6J	0, 50, 250
Holm et al.	2016	C57BL/6J, CD-1	0, 50, 150
Saad et al.	2016	CD-1	0 & 150
Hay-Schmidt et al.	2017	C57BL/6J	0, 50, 150
Philippot et al.	2017	NMRI	0, 60
Zhao et al.	2017	C57BL/6	0, 100
Hurtado-Gonzalez et al.	2018	CD-1 nude xenograft	0, 20 (3 times daily)
Philippot et al.	2018	NMRI	0, 30, 60
Iacob et al.	2019	-	0, 50
Rossitto et al. (a & b)	2019	CD-1	0, 30
Viswanathan et al.	2019	Swiss	0, 500
Pinto et al.	2020	Dp(16)	0, 100

Philippot et al.	2021	NMRI	0, 60
Philippot et al.	2022	NMRI	0, 60
Harshaw and Warner	2022	C57BL/6J	0, 103.9
Baker et al.	2023	C57BL/6J	0, 150

Table 6. Mouse Studies Reviewed for General and Neurodevelopmental Toxicity. The human-mouse conversion provided in Table 1 indicates a 1g dose in a 60Kg adult (16.6mg/Kg) when converted to a mouse dose produces a HED of ~205mg/Kg. A mouse dose of ~150-200mg/Kg is therapeutic, as reported from ED_{50} experimental studies and calculated using HED conversions, see Human Equivalent Dosages. Several studies report maternal and developmental impacts below this "therapeutic" HED. As described below, IP dosages ≥ 400mg have been reported to produce toxicity in mice, which varies by mouse strain and animal age.

1. Studies Examining the Influence of APAP Exposure on Neurobehavioral Changes.

Several studies investigated whether exposure to APAP during neurodevelopment causes behavioral changes (and some of these studies also investigated accompanying biological changes in the brain).

Ishida 2007

Exposure to APAP in mice has shown a biphasic response in young male mice treated with dosages of 15.1, 69.5, and 302.3mg/Kg.[292] This included increased latency in the Morris water maze with dosing post-training at the high dosage, no change in latency at the middle dosage, and decreased latency at the lowest dosage. The high dosage (302.3mg/Kg) that was reported to increase latency in this study is well below expected hepatotoxic dosages for mice, with mouse-human conversion resulting in an estimated HED of 24.6mg/Kg, which is ~1475mg or ~1.5g in a 60Kg reference human. This HED dose is 1.5X the human therapeutic single dose of 1000mg, and about half of a daily dose of 3000 mg.

Gould 2012

This mouse study examined what effect exposure to APAP had on cortical cannabinoid levels and on behaviors associated with ASD and ADHD.[293] The study authors used three different mouse strains (BTBR, C57BL/6, 129S1) to examine the impact of APAP on social interactions and restrictive-repetitive digging behavior (marble burying). This study reported that 100mg/Kg APAP increased 129S1 social novelty preference and marble burying, but APAP only increased BTBR mouse social interaction but not marble burying. They also reported strains differed in endogenous cannabinoids and serotonergic binding in the brain, with the highest CP55,940 (cannabinoid) binding in C57BL/6 and the highest 8-OH-DPAT (serotonergic) binding in 129S. These results are consistent with variations in genetics modifying responses to endogenous and exogenous chemical interactions.

Viberg 2014

A study performed by Viberg et al. showed that APAP exposure during neurodevelopment resulted in significant changes in social-emotional, activity, and learning behaviors as well as changes in the levels

[292] Ishida et al. Effect of acetaminophen, a cyclooxygenase inhibitor, on Morris water maze task performance in mice. J Psychopharmacol. 2007 Sep;21(7):757-67. doi: 10.1177/0269881107076369. Epub 2007 Jul 2. PMID: 17606472.
[293] Gould et al. Acetaminophen differentially enhances social behavior and cortical cannabinoid levels in inbred mice. Prog Neuropsychopharmacol Biol Psychiatry. 2012 Aug 7;38(2):260-9. doi: 10.1016/j.pnpbp.2012.04.011. Epub 2012 Apr 21. PMID: 22542870; PMCID: PMC3389197.

of brain derived neurotropic factor (BDNF). [294] Brain-derived neurotrophic factor (BDNF) plays a crucial role in mammalian brain development, including neuronal survival and differentiation, neuronal growth, connectivity, plasticity, and overall cognitive processes.[295] This study used NMRI mice and demonstrated changes in the brain when young pups (10-day old males and females) were exposed to a single 30mg/Kg or repeated (30+30mg/Kg) dosing of APAP, with the two 30mg/Kg doses separated by 4 hours. The administration of the drug on post-natal day 10 (PND10) corresponds to exposing a human fetus in the third trimester. The study showed that the concentration of APAP decreased by 96.5% between 1-2 hours after exposure, and the second exposure to 30mg/Kg resulted in a return to brain concentrations that were not significantly different from the initial dosing (4.95ug/g versus 4.86ug/g). The second dose in mice models a therapeutic treatment to maintain APAP brain concentrations due to the rapid clearance of APAP in NMRI mice. This 30mg/Kg dose corresponds to a relatively low HED of about 146mg in a 60Kg human.

The study demonstrated differential changes in brain derived neurotrophic factor (BDNF) between controls and subjects treated with 30+30mg/Kg. BDNF was elevated in the frontal cortex and decreased in the parietal cortex.

The study also demonstrated that APAP exposure caused significant changes in behavior across multiple tests, including altered spontaneous behavior, decreased habituation capability, and reduced spatial learning and working memory. Single dose mice showed comparable results to controls but mice dosed twice showed increased horizontal locomotion at all three times tested, increase vertical rearing at all three times tested, and decreased general activity at 0-20 minutes, and increased general activity at 20-40 and 40-60 minutes, determined by beam brakes and cage movement vibrations. APAP exposure also resulted in learning or memory deficits, as indicated in the radial arm maze, such that single dose mice showed comparable results to controls, but mice subject to a second dose had increased errors and took significantly longer finding all eight food pellets on both the second and third days of testing. Moreover, while untreated and single treatment APAP exposed mice showed increasing improvements in speed and decreased reductions in errors on consecutive days 2 and 3, the mice exposed to a second dose of APAP showed no improvements or differences in adaptive behavior between days 2 and 3. When these animals were treated with APAP 30 minutes before performing hot plate testing or elevated plus maze testing, those mice with a single dose showed differences in hot plate testing and time spent on open arms compared to controls, and the mice that had previously received two doses as pups showed significant differences from both controls and single dose mice.

Saad 2016

Saad et al. used the mouse model to test the observed association between maternal intake of APAP and attention deficit hyperactivity disorder (ADHD) in their offspring. The authors proposed that APAP exposure during pregnancy would result in hyperkinetic dysfunctions in offspring.[296] The authors did not report elevated liver enzyme (ALT) with oral dosing of 150mg/Kg in mice, which they referred to as the equivalent to the FDA-approved maximum human clinical dose. The study authors concluded that their

[294] Viberg et al. Paracetamol (acetaminophen) administration during neonatal brain development affects cognitive function and alters its analgesic and anxiolytic response in adult male mice. Toxicol Sci. 2014 Mar;138(1):139-47. doi: 10.1093/toxsci/kft329. Epub 2013 Dec 21. PMID: 24361869.

[295] Colucci-D'Amato et al. Neurotrophic Factor BDNF, Physiological Functions and Therapeutic Potential in Depression, Neurodegeneration and Brain Cancer. Int J Mol Sci. 2020 Oct 21;21(20):7777. doi: 10.3390/ijms21207777. PMID: 33096634; PMCID: PMC7589016.

[296] Saad et al. Is There a Causal Relation between Maternal Acetaminophen Administration and ADHD? PLoS One. 2016 Jun 13;11(6):e0157380. doi: 10.1371/journal.pone.0157380. PMID: 27295086; PMCID: PMC4905664.

study "refutes that hypothesis that prenatal exposure to APAP causes hyperkinetic dysfunction in mouse offspring." But this study includes results supporting that hypothesis. There were four findings in the behavioral analysis with P<0.05, but due to the number of tests performed, P<0.008 was considered statistically significant. The summary table reports mean values for changes in locomotor activity, and this table is devoid of P-values, but enough data is provided to estimate each (two-tailed P value), which results in TLMA (P=0.0184), Rear (P=0.0652), Central (P=0.3843), Peripheral (P=0.0116), Fine (P=0.0086), Total Ambulation (P=0.0296). The authors indicate, "Mice displaying ADHD-like behavior were expected to have greater total locomotor activity, ambulation, and fine repetitive movements..." (p 8/10). There are modest changes in the average activity (~8-9% differences) of the mice, consistent with the hypothesis, and the individual results have low probabilities of occurring by chance. Moreover, if Total Ambulation is tested (P<0.05) alone as a summary statistic, it results in rejecting the null hypothesis, and indicates that APAP causes hyperkinetic dysfunction in mouse offspring. In fact, this is the case for total locomotor activity (P=0.0184), ambulation (P=0.0296), and fine repetitive movements (P=0.0086), but performing these three "expected" results and assuming a=0.05, the P value is adjusted to 0.0167 and only fine repetitive movements are statistically significant, while testing all six measurements with the adjusted P value of 0.0083, and there are no statistically significant findings.

The following principles, published by the ASA,[297] provide some insight on interpretation of these data.

1. P-values can indicate how incompatible the data are with a specified statistical model.
2. P-values do not measure the probability that the studied hypothesis is true, or the probability that the data were produced by random chance alone.
3. Scientific conclusions and business or policy decisions should not be based only on whether a p-value passes a specific threshold.
4. Proper inference requires full reporting and transparency.
5. A p-value, or statistical significance, does not measure the size of an effect or the importance of a result.
6. By itself, a p-value does not provide a good measure of evidence regarding a model or hypothesis.

Regarding effect size,

> Statistical significance is not equivalent to scientific, human, or economic significance. Smaller p-values do not necessarily imply the presence of larger or more important effects, and larger p-values do not imply a lack of importance or even lack of effect.

Based on these principles, the results from this study that show P<0.05 but not P<0.008 should be interpreted under "larger p-values do not imply a lack of importance or even lack of effect."

Zhao 2017; Zhao 2019

Zhao et al. (2017)[298] examined the interaction of APAP and lipopolysaccharide (LPS). Adult male (10-11 weeks old) C57BL/6 mice were treated with APAP (100mg/Kg/day) half an hour prior to intracerebroventricular microinjection of LPS every day thereafter until the end of the study period. Mice treated with LPS exhibited impaired performance in the Morris water maze without changing spontaneous

[297] Ronald L. Wasserstein & Nicole A. Lazar (2016) The ASA Statement on p-Values: Context, Process, and Purpose, The American Statistician, 70:2, 129-133, DOI: 10.1080/00031305.2016.1154108

[298] Zhao et al. Acetaminophen attenuates lipopolysaccharide-induced cognitive impairment through antioxidant activity. J Neuroinflammation. 2017 Jan 21;14(1):17. doi: 10.1186/s12974-016-0781-6. PMID: 28109286; PMCID: PMC5251335.

locomotor activity. APAP suppressed the accumulation of pro-inflammatory cytokines and microglial activation induced by LPS in the hippocampus. LPS is also reported to induce fevers in exposed mice,[299] so mitigation of LPS cognitive impairment may also be due to the fever-reducing/antipyretic activity of APAP. These results were also inverted at different dosages by this same group. Zhao et al (2019)[300] also used young male (6-8 weeks old) C57BL/6 mice and reported that LPS reduces oxidative stress caused by APAP by upregulating the expression of hepatic GSH and reducing MDA levels. Specifically, pretreatment with LPS reversed the effects of APAP toxicity in mice (300mg/Kg) and rats (1000 mg/Kg). The proposed mechanism was based on TNF signaling, such that the amount of TNF- in the liver was increased after LPS, but there was no increase in the production of hepatic GSH in rats lacking TNF- or the receptor (TNFR1).

Hay-Schmidt 2017

Hay-Schmidt et al. performed a study on C57BL/6 male mice exposed to APAP *in utero* (150mg/Kg) and reported that reductions in male sexual behavior were associated with a decrease in the number of neurons in the sexually dimorphic nucleus (SDN) of the preoptic area (POA) in the anterior hypothalamus.[301] Regarding HED dosage,

> The higher dose of 150 mg/kg/day was chosen so it also was in the range of the human exposure when a system of allometry based on the body surface area difference between mouse and human was applied dividing the mice dose with a factor of 12.33….

Exposed males exhibited changes in urinary marking behavior as adults and had a less aggressive territorial display towards other males. During sex with females in oestrus, exposed males did not ejaculate (~0%) during intromission in contrast to controls (~50%). These data show that exposure to APAP alters male rat brains and sexual behavior in adulthood. The authors concluded that there is growing evidence that suggests the need to limit the use of APAP by pregnant women.

Philippot 2017

This study found that when mice were exposed to APAP during a critical period of brain development, it had adverse effects on behavior.[302] Male and female mice were exposed to APAP (0, 60mg/Kg) on PND 3, 10 or 19. The authors report that PND 3 corresponds to about the beginning of the third trimester in human gestation, PND 10 is the time around birth in humans, and PND 19 is about age 2 in humans. This is supported by numerous studies, reviewed by other investigators, comparing rodent and human brain development.[303] The authors report that PND 3 was selected as a point preceding the brain growth spurt

[299] Kozak et al. Lipopolysaccharide induces fever and depresses locomotor activity in unrestrained mice. Am J Physiol. 1994 Jan;266(1 Pt 2):R125-35. doi: 10.1152/ajpregu.1994.266.1.R125. PMID: 8304533.
[300] Zhao et al. Lipopolysaccharide protects against acetaminophen-induced hepatotoxicity by reducing oxidative stress via the TNF-α/TNFR1 pathway. Biochem Biophys Res Commun. 2019 Jun 4;513(3):623-630. doi: 10.1016/j.bbrc.2019.03.181. Epub 2019 Apr 11. PMID: 30981501.
[301] Hay-Schmidt et al. Prenatal exposure to paracetamol/acetaminophen and precursor aniline impairs masculinisation of male brain and behaviour. Reproduction. 2017 Aug;154(2):145-152. doi: 10.1530/REP-17-0165. Epub 2017 May 30. PMID: 28559473.
[302] Philippot et al. Adult neurobehavioral alterations in male and female mice following developmental exposure to paracetamol (acetaminophen): characterization of a critical period. J Appl Toxicol. 2017 Oct;37(10):1174-1181. doi: 10.1002/jat.3473. Epub 2017 Apr 27. PMID: 28448685.
[303] Semple et al. Brain development in rodents and humans: Identifying benchmarks of maturation and vulnerability to injury across species. Prog Neurobiol. 2013 Jul-Aug;106-107:1-16. doi: 10.1016/j.pneurobio.2013.04.001. Epub 2013 Apr 11. PMID: 23583307; PMCID: PMC3737272.

(BGS), PND 10 would be the peak of BGS, and PND 19 would be at the end of BGS. Spontaneous behavior was observed at the age of 2 months when introduced to a new home environment. There were adverse effects on adult behavior and cognitive function when male and female mice were exposed to APAP on PND 3 and 10, but not on PND 19. Exposure to APAP during these times in neurodevelopment resulted in a deficit in habituation capability (as measured by both horizontal locomotion and rearing), including hypoactivity when first introduced to the new environment and hyperactivity in both horizontal and vertical planes after 40 minutes. The authors report,

> exposure to paracetamol on PND 3 and 10, but not on 19, affects spontaneous behavior and decreases habituation capability in a novel home environment in adult mice.

Philippot 2018

This study examined if the cannabinoid receptor (CB1R) and APAP can interact when exposure occurs on PND 10.[304] WIN 55,212-2 (WIN) was used as an agonist for CB1R (Ki = 1.9 nM). The male mice were exposed to different combinations of WIN and APAP (30mg/Kg versus 30mg/Kg twice, separated by 4hrs). When compared with controls and single agent exposed mice, adult mice showed altered spontaneous behavior and habituation capability in a novel home cage. These adverse effects were associated with reduction of transcript levels of hippocampal synaptophysin (Syp) and tropomyosin receptor kinase B (Trkb), and cerebral cortical fatty acid amide hydroxylase (Faah) 24 hours post exposure. As indicated previously, FAAH produces the APAP metabolite AM404, which modifies cannabinoid and serotonergic signaling. The study supports the hypothesis that APAP and WIN can interact when exposure occurs during brain development in mice. The authors indicate that these results raise concerns about the safety of APAP because it is a recommended drug during pregnancy and early life.

Philippot 2022

This 2022 study evaluated the impact of PND 10 exposure to APAP (0, 60mg/Kg) on behaviors and on biological changes relevant to ASD and ADHD in humans.[305] The study authors examined spontaneous behavior, habituation, memory, learning, and cognitive flexibility. They examined markers of oxidative stress, axon outgrowth, and glutamatergic transmission in the hippocampus. In home cage testing, mice that were neonatally exposed to APAP displayed impaired habituation capability, reduced memory and learning ability, and decreased cognitive flexibility in adulthood. Results on the Morris water maze showed APAP exposure also resulted in reduced memory, learning and cognitive flexibility compared to control mice. These effects were associated with increased hippocampal oxidative stress, as indicated by increased Nrf2. The authors conclude that APAP can have negative effects on brain development and induces acute oxidative stress in the hippocampus at HEDs. They conclude,

> Control animals reached base-line activity following 90 min in the new home cage. Mice that neonatally were exposed to APAP displayed an increased activity during the last 30 min of recording compared to these controls. Habituation is considered a non-associative form of learning, and hence is considered a cognitive function (Groves and Thompson, 1970; Daenen et al., 2001; Wright et al., 2004). Previous findings following developmental exposure to APAP in

[304] Philippot G, Hallgren S, Gordh T, Fredriksson A, Fredriksson R, Viberg H. A Cannabinoid Receptor Type 1 (CB1R) Agonist Enhances the Developmental Neurotoxicity of Acetaminophen (Paracetamol). Toxicol Sci. 2018 Nov 1;166(1):203-212. doi: 10.1093/toxsci/kfy199. PMID: 30165669.

[305] Philippot et al. Paracetamol (Acetaminophen) and its Effect on the Developing Mouse Brain. Front Toxicol. 2022 Mar 22;4:867748. doi: 10.3389/ftox.2022.867748. PMID: 35391823; PMCID: PMC8981466.

> rodents have also indicated both a hypo-responsiveness to a new environment and effects on memory and learning (Blecharz-Klin et al., 2017; Philippot et al., 2017; Philippot et al., 2018). In line with this observation, it has been shown that ASD patients display hypo-responsiveness to novel stimuli (van Engeland et al., 1991; Webb et al., 2010).

Harshaw 2022

Harshaw showed that APAP (0, 103.9mg/Kg) exposure, 1 dose every 3 days (PND 5, 8, and 11), during brain development caused long term effects including impaired social-emotional and repetitive behaviors.[306] The study authors proposed that most of the earlier studies did not take into account that APAP exposure often happens in the context of immune activation and fever. To better mimic the repeated infections that are common in infancy, researchers used the cytokine interleukin-1β (IL-1β) to induce immune activation three times during early postnatal development in mice. On those days, the mice were given either IL-1β or a saline solution, followed by either APAP or a vehicle. The mice were then subjected to a series of tests of social-emotional and repetitive behavior. The researchers found several long-term effects of the IL-1β and APAP treatments, including shifts in repetitive behavior and emotional hyperthermia following IL-1β and increased social caution in male mice following APAP. There were also significant interactions between IL-1β and APAP, with females displaying greater anxiety-related behavior and males showing elevated levels of avoidance of unfamiliar social partners. These findings suggest that IL-1β-induced inflammation and APAP have both distinct effects and significant interactions during early life, with long-lasting effects in causing behaviors that are consistent with neurodevelopmental disorders.

Baker 2023

Baker et al looked at pregnant mice that were given APAP at relevant human therapeutic levels (150mg/Kg, GD4-10 through PND 14, PO tablets).[307] The study authors investigated offspring for attention deficits and explored the underlying mechanisms using RNA sequencing for gene expression analysis. Pregnant mice were randomly assigned to receive either acetaminophen or a control substance. An acetaminophen dose of 150 mg/Kg corresponds to a human equivalent dose of about 732 mg in a woman weighing 60Kg (i.e., less than the therapeutic human dose of 1000 mg). The offspring were evaluated using a battery of behavioral tests. The study found that *in utero* APAP exposure led to behavioral changes including increased and hastened separation-induced pup vocalizations, decreased ambulation, and decreased vertical rearing in male adult offspring. Additionally, APAP treatment also resulted in biological changes in the brains of offspring. These included altered prefrontal cortex gene expression related to various molecular pathways. The authors conclude that these findings provide further evidence for the potential neurodevelopmental harm of prenatal APAP exposure. They conclude,

> In conclusion, 150 mg/kg/day APAP exposure during gestation and lactation was associated with increased separation-induced pup vocalizations and reduced exploratory behavior in the open field. Exposure was also associated with gene expression changes in offspring prefrontal cortex indicative of increased DNA damage and altered endocrine and immune system activity. Future

[306] Harshaw and Warner. Interleukin-1β-induced inflammation and acetaminophen during infancy: Distinct and interactive effects on social-emotional and repetitive behavior in C57BL/6J mice. Pharmacol Biochem Behav. 2022 Oct;220:173463. doi: 10.1016/j.pbb.2022.173463. Epub 2022 Sep 11. PMID: 36100070.

[307] Baker et al. Sex-specific neurobehavioral and prefrontal cortex gene expression alterations following developmental acetaminophen exposure in mice. Neurobiol Dis. 2023 Feb;177:105970. doi: 10.1016/j.nbd.2022.105970. Epub 2022 Dec 19. PMID: 36549432; PMCID: PMC9940030.

studies are needed to explore whether the potential mechanisms revealed by RNA-sequencing directly link perinatal APAP exposure with behavior changes.

2. Additional mouse studies examining whether APAP causes neuro-biological damage.

Ruepp 2002; Stamper 2011

Gene transcription and protein translation changes are dynamic and highly orchestrated to respond to developmental cues, endogenous signals, and environmental or pharmaceutical exposures. Numerous investigators have examined the impact of APAP on specific genes or proteins, but there is no single gene or protein identified as impacted by APAP or the known metabolites. Genomic and proteomic approaches have also been performed, and these datasets provide a broader analysis of the oxidative impact of APAP exposure. A study by study by Ruepp et al (of AstraZeneca) used this approach to test APAP toxicity in CD-1 mice:[308]

> "we administered APAP at subtoxic (150 mg/kg ip) and toxic (500 mg/kg ip) doses to overnight fasted mice."

This study reported reduction in hepatic GSH at the low dose (150mg/Kg) with minor changes in pathology at 120 minutes. The high dose (500mg/Kg) resulted in pathology including hepatocyte vacuolation, swelling, sinusoidal narrowing and congestion, with necrosis in the centrilobular zone under light microscopy. These observations were followed up by electron microscopy, and images show mitochondrial swelling at 30 minutes, with enlargement and fusion of mitochondria at 500mg/Kg. Changes in gene expression and protein expression were reported on mitochondrial proteins and stress related responses, but the authors largely reject gene regulation as the primary change due to APAP toxicity. Specifically, the authors report,

> "The decrease of ATP synthase subunits and b-oxidation pathway proteins indicated a loss of energy production. As the morphology of mitochondria was also affected very early at top dose, we concluded that APAP toxicity was a direct action of its known reactive metabolite NAPQI, rather than a consequence of gene regulation."

Recent studies using proteomic analysis on C57BL/6 mice have drawn similar conclusions, suggesting APAP may downregulate P450 enzymes to reduce oxidative damage by APAP exposures and upregulate electron transport chain protein in an attempt to restore ATP production.[309]

Markovic 2013

This study examined the issue of a neonate's access to APAP *in utero* and demonstrated that APAP exposure results in DNA damage consistent with genotoxicity as well as oxidative damage.[310] The study authors report that APAP results in an increased frequency of micronucleus formation in pups transplacentally exposed to APAP at 60mg/Kg. This is a relatively low HED of 4.9mg/Kg or ~300mg,

[308] Ruepp SU, Tonge RP, Shaw J, Wallis N, Pognan F. Genomics and proteomics analysis of acetaminophen toxicity in mouse liver. Toxicol Sci. 2002 Jan;65(1):135-50. doi: 10.1093/toxsci/65.1.135. PMID: 11752693.
[309] Stamper et al. Proteomic analysis of acetaminophen-induced changes in mitochondrial protein expression using spectral counting. Chem Res Toxicol. 2011 Apr 18;24(4):549-58. doi: 10.1021/tx1004198. Epub 2011 Feb 18. PMID: 21329376; PMCID: PMC3079806.
[310] Markovic et al. Lipid peroxidation, detoxification capacity, and genome damage in mice after transplacental exposure to pharmaceutical drugs. Braz J Med Biol Res. 2013 Dec;46(12):1014-1020. doi: 10.1590/1414-431X20132814. Epub 2013 Dec 10. PMID: 24345909; PMCID: PMC3935272.

which is less than one regular strength Tylenol (325mg), or less than half the recommended dose of two tablets (650mg). The reported micronucleus formation is a result of DNA fragmentation or chromosomal instability and is an indication of genotoxicity. This study also reported that APAP resulted in decreased GSH-Px and malondialdehyde (MDA). Collectively, these data are consistent with APAP induced oxidative damage, reduced antioxidant capacity, and DNA damage in transplacentally exposed newborn pups. The difference between Gould et al. and Markovic et al. may in part be explained by Markovic et al. using a different strain of mice, BALB/c. Another difference which is also consistent with Wilson's Principles is timing or the critical window of exposure. More specifically, Markovic et al. was one of the first studies to examine *in utero* gestational exposure to APAP, and these exposures occurred relatively late in pregnancy, GD 12-14, since mice normally come to term around 19-20 days. These data were also collected days after the last APAP treatment and hours (6-18 hours) after delivery, such that the reported genetic and oxidative damage detected in the newborn appears longer lasting than the maternal APAP exposure.

Thiele 2015

Thiele et al examined the impact of exposure to 50 or 250 mg/kg (IP) APAP on C57Bl/6J females on gestation day 12.5 and reported immune impacts that damaged the placenta and impaired fetal development.[311] The 250mg/Kg dosage produced transient increases in ALT and AST with associated hepatotoxicity, but this was not observed at 50mg/Kg. The majority of findings were specific to the 250mg/Kg dosage, but there was significantly altered placental histology, reduced fetal weight, and reduced hematopoietic stem cells at 50mg/Kg.

Hurtado-Gonzalez 2018

A study by Hurtado-Gonzalez et al. investigated the potential effects of APAP on fetal gonadal development during pregnancy.[312] Using *in vitro* and xenograft approaches, the study exposed first and second-trimester human fetal testes/ovaries to therapeutic doses of APAP to examine their impact on germ cell development. They also used a human GC tumor-derived cell line and *in vivo* rat models to determine the mechanism of action of the analgesics. The study found that gonocyte (TFAP2C+) number was significantly reduced relative to controls in first-trimester human fetal testes exposed in vitro to acetaminophen (-28%), and in ovaries exposed to acetaminophen (-43%). Furthermore, in second-trimester human fetal testes xenografted into mice, acetaminophen exposure reduced gonocyte number by 17% and 30% after 1 or 7 days of treatment, respectively. The study also observed gene expression changes in rat fetal testis/ovary cultures and in fetal testes and ovaries after *in vivo* exposure of pregnant rats. These changes included alterations in the expression of genes that regulate DNA/histone methylation and GC pluripotency genes. Expression of the epigenetic regulator TET1, was increased following exposure to APAP (10-50μM) in human NTera2 cells, rat fetal testis/ovary cultures, and in fetal testes and ovaries after *in vivo* exposure of pregnant rats. The authors conclude that these findings raise concerns about APAP use during human pregnancy and highlight the need for further investigation to understand the risks associated with APAP use during pregnancy.

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[311] Thiele et al. Prenatal acetaminophen affects maternal immune and endocrine adaptation to pregnancy, induces placental damage, and impairs fetal development in mice. Am J Pathol. 2015 Oct;185(10):2805-18. doi: 10.1016/j.ajpath.2015.06.019. Epub 2015 Aug 5. PMID: 26254283.

[312] Hurtado-Gonzalez et al. Effects of Exposure to Acetaminophen and Ibuprofen on Fetal Germ Cell Development in Both Sexes in Rodent and Human Using Multiple Experimental Systems. Environ Health Perspect. 2018 Apr 16;126(4):047006. doi: 10.1289/EHP2307. PMID: 29665328; PMCID: PMC6071829.

Pinto 2020

This study examined microglia in the brain of Down syndrome mice and humans and observed that microglia were overactive and causing inflammation in the brain.[313] This led to cognitive problems in the mice, such as decreased spine density and activity of neurons. However, when the defective microglia were removed or treated with APAP (IP, 100mg/Kg/day, PND 20-22), the cognitive problems in the mice were improved. As indicated by Philippot et al (2017), PND 19 was outside the critical window of exposure for APAP. The authors propose that this later window of exposure to APAP could potentially lead to new treatments for cognitive disabilities in people with Down syndrome.

Philippot 2021

This 2021 study continued these study authors' examination of the effects of APAP during the critical window corresponding to third trimester human development.[314] In this study, they examined dentate gyrus (DG), which is important for learning and memory processing. The authors investigated if the cytoarchitecture of the DG is affected. They reported that there were no significant changes in markers for differentiation or migration in the hippocampus after APAP exposure. They also suggest that the DG thickness and area should be evaluated in studies with larger sample sizes, because the limited number of animals (n = 3) for each exposure group determined large effect sizes on DG thickness, but this was coupled to low precision due to limited animals, as indicated by relatively wide 95% CIs overlapping between the means in control groups and exposed groups.

3. Mouse Studies Examining Whether APAP Causes Glutathione Depletion and Toxicity

A number of studies have demonstrated a mechanism of action for APAP toxicity in mice that is also found in humans. In the presence of CYP2E1, APAP metabolizes into the metabolite NAPQI. NAPQI is a highly reactive and toxic metabolite that forms covalent bonds with nucleophilic sites on proteins, particularly cysteine residues. This covalent binding can modify the structure and function of proteins, interfere with enzyme function, impair mitochondrial respiration, and result in additional free radical generation, cellular dysfunction, and apoptosis. Glutathione (GSH) is needed to eliminate the NAPQI. If poor nutrition, pregnancy, or other oxidative stress has reduced glutathione levels, then the APAP-NAPQI and associated metabolite toxicity increases, spreading across more cells and tissues.

Nebert 1976

In 1976, Nebert et al. reported that APAP administration (250, 500, 1000mg/Kg) resulted in both maternal and fetal reductions of total liver glutathione.[315] Maternal glutathione content was reported to decrease by ~15% of normal 2 hr after a 250 mg/kg dose but returned to 63% and 120% of normal after 3 hr and 5 hr, respectively. Both 500 and 1000 mg/kg dosages of APAP were reported to deplete glutathione content to less than 10% of normal within 2 hr, with no recovery during the next 3 hr, causing hepatic necrosis and death in most of these pregnant mice within 12 hrs. In contrast to the results in maternal liver, no significant glutathione depletion was observed in fetal liver at the 250 mg/kg dose of acetaminophen, and there is

[313] Pinto et al. Rescuing Over-activated Microglia Restores Cognitive Performance in Juvenile Animals of the Dp(16) Mouse Model of Down Syndrome. Neuron. 2020 Dec 9;108(5):887-904.e12. doi: 10.1016/j.neuron.2020.09.010. Epub 2020 Oct 6. PMID: 33027640; PMCID: PMC7736620.

[314] Philippot et al. Evaluation of the dentate gyrus in adult mice exposed to acetaminophen (paracetamol) on postnatal day 10. Int J Dev Neurosci. 2021 Feb;81(1):91-97. doi: 10.1002/jdn.10079. Epub 2021 Jan 12. PMID: 33222217.

[315] Nebert et al. Genetic aspects of toxicity during development. Environ Health Perspect. 1976 Dec;18:35-45. doi: 10.1289/ehp.761835. PMID: 829488; PMCID: PMC1475294.

only ~50% depletion at 5 hr with the 1000 mg/kg dose. This study indicates that at APAP dosages that can produce maternal hepatotoxicity, there is reduction of GSH, but no reported hepatoxicity or associated liver necrosis in the developing embryo or fetus. These data are also consistent with the observed resistance to APAP hepatotoxicity in both young mice and rats reported by Mancini et al. (Table 2).

Alhava 1978; Mancini 1980

In a study performed by Alhava et al. in NMRI mice, mice that were 7-9 days old were found to be more sensitive to toxic-lethal dosages of APAP. Specifically, only the 850mg/Kg dosage was toxic to adult mice, but dosages of 700 and 500 were both shown to be toxic to 7-9- or 16-19-day old mice.[316] These differences appear as of function of two variables, the different mouse strains used and the variation in vehicle concentration. The vehicle in Alhava et al., 50% propylene glycol, was noted to produce hypothermia in these mice, but undilute propylene glycol was used by Mancini et al, heated to 40C.[317] Propylene glycol is now recognized to be a CYP2E1 competitive antagonist.[318]

Larrey 1986

A study by Larrey et al. in 1986 showed that pregnant mice are more susceptible to the effects of APAP compared to non-pregnant mice.[319] This study showed that pregnant mice have lower glutathione levels when exposed to APAP compared to non-pregnant animals exposed to APAP. In addition, the lethal dose for pregnant mice was about half of the lethal dose for non-pregnant mice. This study found that pregnant mice had an increased incidence of liver necrosis compared to non-pregnant animals, increased mortality, and glutathione was also shown to be lower in pregnant animals exposed to APAP compared to exposed non pregnant animals (Figure 26, Right). For comparison, the increased toxicity of APAP shows an ~LD50 of 400mg/Kg in pregnant female mice (Figure 26, Center) versus an estimated LD50 of 800mg/Kg (see Table 2) in adult mice (males and females).

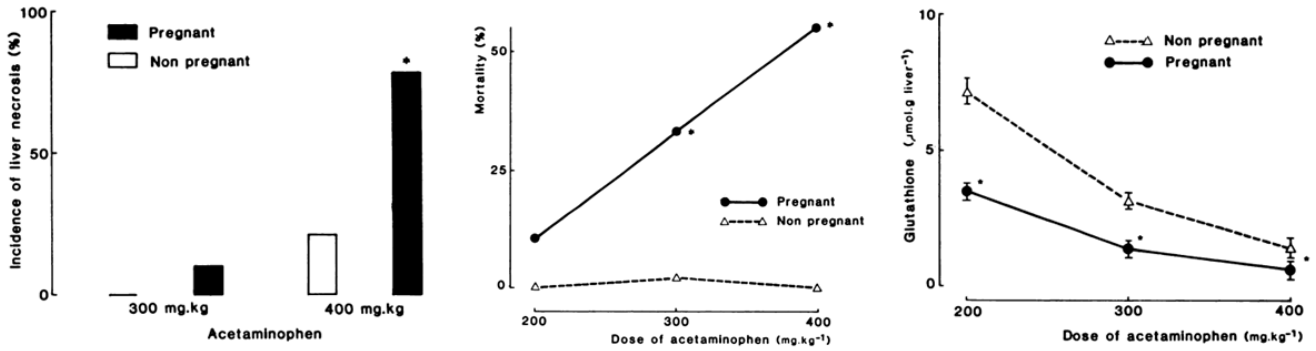

Figure 26. Pregnancy Increases Acetaminophen (APAP) Toxicity. (Left) The incidence of liver necrosis increased in pregnant mice compared to non-pregnant animals exposed to the same dosage of APAP. (Center) Dosages of 200-400mg/Kg produced one death at 300mg/Kg in non-pregnant mice, and all dosages resulted in higher mortality

[316] Alhava et al. Toxicity of paracetamol in relation to age in mice. Acta Pharmacol Toxicol (Copenh). 1978 Apr;42(4):317-9. doi: 10.1111/j.1600-0773.1978.tb02208.x. PMID: 580678.
[317] Mancini et al. Developmental susceptibility to acetaminophen toxicity. Res Commun Chem Pathol Pharmacol. 1980 Mar;27(3):603-6. PMID: 7384649.
[318] Ganetsky et al. Effect of excipients on acetaminophen metabolism and its implications for prevention of liver injury. J Clin Pharmacol. 2013 Apr;53(4):413-20. doi: 10.1002/jcph.24. Epub 2013 Feb 22. PMID: 23436315; PMCID: PMC4383763.
[319] Larrey et al. Effects of pregnancy on the toxicity and metabolism of acetaminophen in mice. J Pharmacol Exp Ther. 1986 Apr;237(1):283-91. PMID: 3083096.

in pregnant animals, with 300 or 400mg/Kg showing significant increases in mortality. (Right) Pregnant mice were shown to have significantly reduced liver concentrations of GSH at all dosages tested.[320]

These studies cannot be performed in humans for ethical reasons, but there are reports on APAP pharmacokinetics in pregnant women that have been analyzed to model human non-pregnant females versus pregnant females.[321, 322] These models indicate that NAPQI production is increased during pregnancy.

Population	Unchanged acetaminophen excreted in urine	Acetaminophen glucuronide	Acetaminophen sulfate	NAPQI
Non-pregnant women	7.38 [0.13–13.46]	53.81 [51.16–57.19]	31.10 [28.98–33.32]	7.71 [6.40–9.35]
First trimester pregnant women	8.62 [0.85–15.15]	56.15 [53.41–59.59]	24.20 [22.29–26.20]	11.03 [9.14–13.36]
Second trimester pregnant women	7.43 [0.80–13.34]	62.06 [59.32–65.09]	21.47 [19.86–23.14]	9.03 [7.48–10.98]
Third trimester pregnant women	7.16 [0.32–12.34]	63.91 [61.71–67.18]	20.65 [19.13–22.36]	8.23 [6.82–10.13]

Data are reported as median [interquartile range]

NAPQI N-acetyl-*p*-benzoquinone imine

Table 7. Fraction, expressed as percentage of total paracetamol (molar) dose, of acetaminophen to metabolites.

Ferguson 1990; Adamson 1991

Several studies have reported elevation of ALT in mice exposed to APAP, and this measurement is used to determine liver stress or damage. A study by Ferguson et al. reported that dosages of 100 or 200mg/Kg produced no impact on ALT, but APAP at 300 or 400mg/Kg IP resulted in elevation in ALT (~10-14K U/L) at 8 hours, with 400mg/Kg resulting in an LD40.[323] This study indicates that B6C3F1 mice have similar sensitivity to the pregnant CD1 mice used by Larrey et al. While diet, housing, vehicle, and age may have influenced the sensitivity to APAP, another explanation for this difference that is consistent with Wilson's Principles is the influence of genetic background; the B6C3 strain being an F1 cross of two different mouse strains versus the CD1 mice, another commonly used strain for developmental and reproductive toxicity testing. Regarding age and as indicated previously by Mancini et al. (Table 2),[86] the toxicity of APAP is predominately reported to increase with age and this interaction has been supported independently (Table 8).[324] Adamson et al. reported that this response was not due to lack of P450 enzymes, because faster clearance of NAPQI was reported in young mice. It is also notable that the mice at 2-weeks of age have yet to be weaned, and it is now known that breast milk is a rich source of

[320] Larrey et al. Effects of pregnancy on the toxicity and metabolism of acetaminophen in mice. J Pharmacol Exp Ther. 1986 Apr;237(1):283-91. PMID: 3083096.

[321] Brookhuis et al. Modelling Tools to Characterize Acetaminophen Pharmacokinetics in the Pregnant Population. Pharmaceutics. 2021 Aug 20;13(8):1302. doi: 10.3390/pharmaceutics13081302. PMID: 34452263; PMCID: PMC8400310.

[322] Mian et al. Physiologically Based Pharmacokinetic Modeling to Characterize Acetaminophen Pharmacokinetics and N-Acetyl-p-Benzoquinone Imine (NAPQI) Formation in Non-Pregnant and Pregnant Women. Clin Pharmacokinet. 2020 Jan;59(1):97-110. doi: 10.1007/s40262-019-00799-5. PMID: 31347013; PMCID: PMC6994454.

[323] Ferguson et al. Acetaminophen-induced alterations in pancreatic beta cells and serum insulin concentrations in B6C3F1 mice. Toxicol Appl Pharmacol. 1990 Jun 15;104(2):225-34. doi: 10.1016/0041-008x(90)90297-8. PMID: 2194324.

[324] Adamson et al. Postnatal mice have low susceptibility to paracetamol toxicity. Pediatr Res. 1991 May;29(5):496-9. doi: 10.1203/00006450-199105010-00016. PMID: 1896254.

antioxidants, glutathione peroxidase, and GSH in humans.[325, 326, 327] Similar activity is expected in all mammals, but milk GSH testing is largely unexplored in rodents due to their size. Bovine whey protein has also been shown to protect mice from 300mg/Kg APAP induced hepatotoxicity as indicated by reduced liver enzymes (glutamate pyruvate transaminase, GPT; alkaline phosphatase, ALP), reduced creatinine, increased BUN, increased antioxidant enzymes (catalase, CAT; superoxidase dismutase, SOD; glutathione peroxidase, GSH-Px) and reduced TBARS.[328]

	Lethality†		
		Paracetamol	
Age (wk)	Control	300 mg/kg	500 mg/kg
2	0	0	0
3	0	0	3
8–10	0	0	7
24–26	0	0	8
32–34	0	0	5
52–54	0	3	9

* In each age group, 10 animals received either dose of paracetamol and four control animals received saline intraperitoneally.
† Number of deaths in 8 h.

Table 8. The Lethality of Paracetamol (APAP) in Mice by Age in CD-1 Mice. The number of mice that die out of 10/group increases with dose of APAP IP and age of mice. In two-week-old mice there was no lethality, no hepatocytic necrosis, and no increase in plasma transaminases.

NTP 1993

A continuous dietary exposure toxicity study was performed by the NTP.[329] These studies included 14-day, 13-week, and 2-year exposures to APAP via fed diets at exposures ranging from 800-12,500PPM, 800-25,000PPM, and 600-6000PPM. For comparability, these dosages are 600PM = 0.06% or ~86mg/Kg to 25,000PM = 2.5% or 3575mg/Kg. The upper dose of 3575mg/Kg in mice converts to a human dosage of 291mg/Kg or a 17.5g dose in a 60Kg reference human. This upper dose resulted in one death in a female mouse during the 13-week, and the 12500PPM (mouse 1788mg/Kg, HED 146mg/Kg) dose also resulted in the death in two male mice during the 13-week study. These studies are also consistent with the expected acute toxicity of humans at dosages of 7.5g or ≥150mg/Kg.

Thomson 1995; Kelava 2010

[325] Ankrah et al. Human breastmilk storage and the glutathione content. J Trop Pediatr. 2000 Apr;46(2):111-3. doi: 10.1093/tropej/46.2.111. PMID: 10822938.
[326] Sari et al. Antioxidant capacity of fresh and stored breast milk: is -80°C optimal temperature for freeze storage? J Matern Fetal Neonatal Med. 2012 Jun;25(6):777-82. doi: 10.3109/14767058.2011.592230. Epub 2011 Aug 1. PMID: 21801121.
[327] Miranda et al. Oxidative status of human milk and its variations during cold storage. Biofactors. 2004;20(3):129-37. doi: 10.1002/biof.5520200302. PMID: 15665383.
[328] Athira et al. Ameliorative potential of whey protein hydrolysate against paracetamol-induced oxidative stress. J Dairy Sci. 2013 Mar;96(3):1431-7. doi: 10.3168/jds.2012-6080. Epub 2013 Jan 9. PMID: 23313001.
[329] National Toxicology Program. NTP Toxicology and Carcinogenesis Studies of Acetaminophen (CAS No. 103-90-2) in F344 Rats and B6C3F1 Mice (Feed Studies). Natl Toxicol Program Tech Rep Ser. 1993 Jan;394:1-274. PMID: 12637965.

Additional studies in mice have shown that propylene glycol is protective against APAP hepatoxicity, due to inhibition of CYP2E1, which also results in reduced loss of GSH and lowered ALT.[330,331] These data support that propylene glycol modifies APAP toxicity by decreasing CYP2E1. These data are also consistent with the observations that lower expression or reduced activity of cytochrome P450 enzymes results in increased resistance to APAP, mimicking the Cyp2e1 -/- and Cyp1a2 -/- mutant mice (Figure 27).

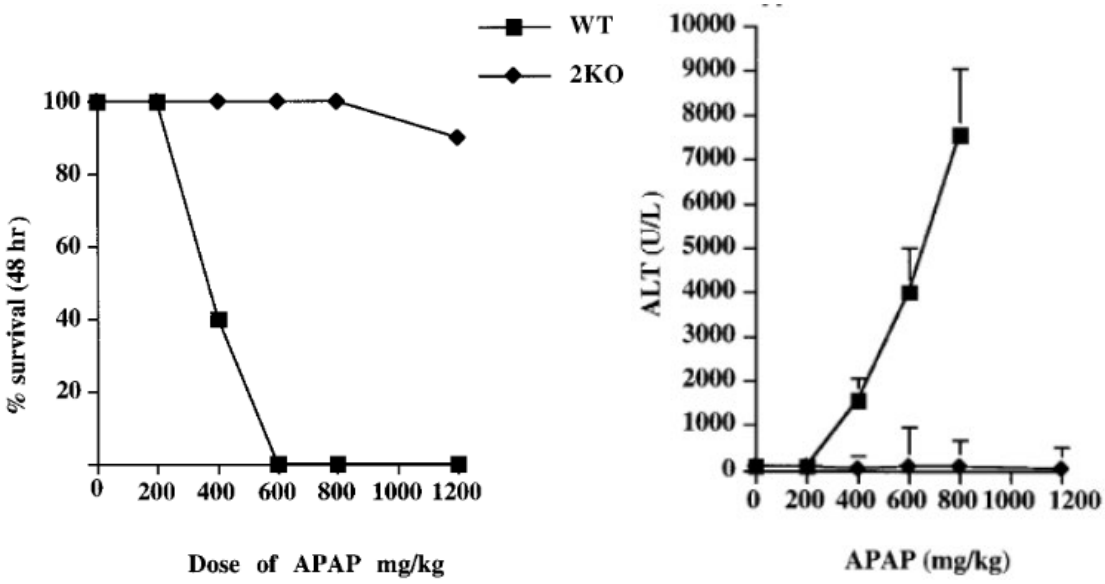

Figure 27. Cytochrome P450 and Acetaminophen (APAP) Toxicity. (Left) Survival of wild-type and double-null mice (Cyp2e1 -/- and Cyp1a2 -/-) after APAP. Mice were treated with increasing doses of APAP (n = 8 per group). Survival was observed 48 h after the challenge dose and percent survival was plotted as a function of challenge dose. (Right) ALT in APAP treated mice. ALT was measured in plasma of wild-type and double-null mice after APAP exposure. Values are means +/- SE from groups of four to six mice. *p <= 0.05 WT vs 2KO mice.[332]

Patel 2001

Patel examined acetaminophen in C57BL/6 mice, giving three dosages IP at 100, 200, or 250mg/Kg results in no significant increase in liver weight or ALT.[333] There were also no significant changes reported in uterine weight, uterine peroxidase activity, or uterine or hepatic progesterone receptor with APAP treatment compared to vehicles. These data indicate that at IP dosages up to 250mg/Kg, for three consecutive days, in C57Bl/6 female mice there is no overt hepatotoxicity. For comparison, the HED "therapeutic" dose of APAP in mice is 205mg/Kg, so these dosages are ~0.5X, ~1X, and ~1.2X HEDs.

[330] Kelava et al. Influence of small doses of various drug vehicles on acetaminophen-induced liver injury. Can J Physiol Pharmacol. 2010 Oct;88(10):960-7. doi: 10.1139/y10-065. PMID: 20962895.
[331] Thomsen et al. Cytochrome P4502E1 inhibition by propylene glycol prevents acetaminophen (paracetamol) hepatotoxicity in mice without cytochrome P4501A2 inhibition. Pharmacol Toxicol. 1995 Jun;76(6):395-9. doi: 10.1111/j.1600-0773.1995.tb00168.x. PMID: 7479582.
[332] Zaher et al. Protection against acetaminophen toxicity in CYP1A2 and CYP2E1 double-null mice. Toxicol Appl Pharmacol. 1998 Sep;152(1):193-9. doi: 10.1006/taap.1998.8501. PMID: 9772215.
[333] Patel and Rosengren. Acetaminophen elicits anti-estrogenic but not estrogenic responses in the immature mouse. Toxicol Lett. 2001 May 31;122(1):89-96. doi: 10.1016/s0378-4274(01)00352-6. PMID: 11397560.

This study is consistent with an absence of hepatotoxicity with mouse dosages near recommended therapeutic HED.

Leroux 2010

Time and/or dose dependent toxicity was reported by Leroux et al.[334] This study reports that a dose of 10ug given PD1-5 resulted in 38% survival (6/16). Conversion of this dosages is a tenuous estimate, as the dose was not adjusted by pup weight or age, so a PD1 newborn, which is expected to weigh ~1g is given the same dose of a PD5, reported to weigh 2.4 to 3.6 g. This results in newborn pups being treated with 2.4-3.6X the dosage of the PD5, so estimated dosages range from 100mg/Kg for the 100ug dose at PD1, assuming a 1-gram pup, to 2.7mg/Kg for the 10ug dose in a 3.6-gram PD5 pup. The high APAP dose (100ug), given early PD1-5, resulted in increased pup lethality. The low dose of APAP (10ug), given to older pups (PD5-10) was reported to protect the brain, as indicated by reduced sizes of white matter lesions with ibotenate exposure.

Iacob 2019

This study exposed pregnant white mice exposed to acetaminophen (50mg/Kg/day) from GD5-7.[335] After delivery, pups were examined for liver and kidney pathology. Results showed that the number of fetuses born was normal. Pathology of the liver and kidney revealed APAP produced urinary tube necrosis, vascular congestions, and vascular disorders in the kidneys, and vascular congestions, vascular disorders, and hemosiderin deposits in Kupffer cells in the liver. The study concluded that APAP did not reduce fertility at this dose and duration, but it did produce microscopic changes in the liver and kidney pathology in offspring.

Viswanathan 2019

Viswanathan et al[336] gave mice different combinations of a Centella asiatica methanol (CAM) extract and APAP (PO, 500mg/Kg) and measured the levels of oxidative and nitric oxide stress in the brains of mice. The authors also looked at the cytotoxicity and inflammation levels by examining the expression of cytokine mediators in the brain tissue and assessing apoptosis/necrosis. The results showed that APAP decreased ferric reducing antioxidant power and increased reactive nitrogen species in brains both *in vitro* and *in vivo*. CAM extract was able to reduce this effect and increase the scavenging activity. APAP showed the presence of more double positive necrotic/apoptotic cells in astrocytes. The extract was also able to inhibit the expression of pro-inflammatory cytokines and increase the expression of anti-inflammatory cytokines, which helped to decrease damage to the brain cells. Overall, the study suggests that the CAM extract has antioxidant and anti-inflammatory effects against oxidative stress and inflammation in the brain caused by APAP.

4. Studies Examining APAP and Reproductive Toxicity.

A number of studies have investigated reproductive toxicity of fetal exposure to APAP. There is evidence that exposure to toxicants can result in epigenetic alterations, notably DNA methylation. Three areas of

[334] Leroux et al. Neuroprotective effects vary across nonsteroidal antiinflammatory drugs in a mouse model of developing excitotoxic brain injury. Neuroscience. 2010 May 19;167(3):716-23. doi: 10.1016/j.neuroscience.2010.02.042. Epub 2010 Feb 24. PMID: 20188153.

[335] Iacob et al. Consequences of analgesics use in early pregnancy: Results of tests on mice. Sci Total Environ. 2019 Nov 15;691:1059-1064. doi: 10.1016/j.scitotenv.2019.07.212. Epub 2019 Jul 15. PMID: 31466187.

[336] Viswanathan et al. Protection of mouse brain from paracetamol-induced stress by Centella asiatica methanol extract. J Ethnopharmacol. 2019 May 23;236:474-483. doi: 10.1016/j.jep.2019.03.017. Epub 2019 Mar 11. PMID: 30872170.

increasing regulatory activity are reproductive toxicity, developmental toxicity, and neurotoxicity. These are endpoints for which an exposure threshold is assumed. That is, an acceptable level of exposure, below which no toxicity is predicted, can be calculated. Studies have confirmed that specific chemical exposures may be linked to infertility, neurodevelopmental disorders, carcinogenesis, and reproductive disruption of gametes.

Reel 1992

This study examined the reproductive toxicity of APAP in mice.[337] The study authors examined chronic exposure to APAP in chow at dosages of 0, 0.25, 0.5, and 1.0%, which were estimated to result in APAP administration of 0, 357, 715, and 1430 mg/Kg, respectively. A preliminary dose study was also performed and estimated that the oral dosage of APAP that produced lethality had a reported LD10 (14 days) of 1.81% APAP (~2574mg/Kg/day) and an LD50 (14 days) of 4.11% APAP (~5877mg/Kg/day). The 1% dose was reported to reduce litter per pair, live pup weight, weaning (PN28) weight, and mating weights (PN74+/-10). In F1 offspring, this dose also resulted in reduced body weights in males and females, and increased weights in the liver and brain, that were reported as significantly different in females. The F1 males were also reported to have more than a doubling of the percentages, 7.3% versus 16.4%, of abnormal sperm at the 1% dosage. Significant reductions in weight were also reported in weaning females at 0.25%, in males and females at mating at 0.25%, and in weaning and mating males and females at 0.5% APAP in diet. The conclusion of this study reads,

> "APAP was found to be a reproductive and systemic toxicant in Swiss CD-I mice, albeit at relatively high exposure levels."

While this statement is largely driven by the mg/Kg dosing, it should again be remembered that the 0.25%, 0.5%, and 1% APAP diets can be converted from mouse mg/Kg dose to a HED, and results in dosages of 30.5, 58.1, and 116.3mg/Kg, respectively. The upper dose for a reference 60Kg human, results in a dose of 6975mg, or approximately 7 grams. As expected, this "relatively high exposure" is remarkably near the 7.5g acute toxic dose in humans using the mouse-human conversion (Table 1).

Holm 2015; Holm 2016

In utero exposure to APAP can also impact reproductive cells and tissues in the offspring. A study by Holm et al. (2015) first reported aniline conversion into APAP and also indicated that APAP impacted anal-genital-distance (AGD), consistent with fetal androgen deficiency during external genitalia differentiation.[31] The study also performed testing on NCI-H295R human adrenocortical carcinoma cell line to examine human androgen production *in vitro* and reported APAP was anti-androgenic. Specifically, steroids were analyzed after exposure for 48h to the following concentrations of 0, 0.1, 0.314, 1, 3.14, 10, 31.4, 100, 314, and 1000μM APAP. APAP increased pregnenolone (EC50=146μM; P=.001) and decreased hormone levels downstream from progesterone: 17a-hydroxyprogesterone (EC50=43 μM; P=.04), androstenedione (EC50=289 mM; P=.001) and testosterone (EC50=97μM; P=.007). Holm et al. (2016) also examined the impact of APAP on female offspring with *in utero* exposure to APAP.[338] This study also observed changes in AGD in female offspring, with the majority occurring at the high dose

[337] Reel et al. Reproductive toxicity evaluation of acetaminophen in Swiss CD-1 mice using a continuous breeding protocol. Fundam Appl Toxicol. 1992 Feb;18(2):233-9. doi: 10.1016/0272-0590(92)90051-i. PMID: 1601223.

[338] Holm et al. Intrauterine Exposure to Paracetamol and Aniline Impairs Female Reproductive Development by Reducing Follicle Reserves and Fertility. Toxicol Sci. 2016 Mar;150(1):178-89. doi: 10.1093/toxsci/kfv332. Epub 2016 Jan 5. PMID: 26732887.

(150mg/Kg), but both dosages (50 and 150mg/Kg) resulted in reduced primordial and growing ovarian follicles. The 50mg/Kg is referenced by the authors as the dose allowed to be taken by pregnant women, and this statement is consistent with the HED in mice. APAP exposure was associated with reduced fertility (% full term pregnancies) and reduced litter size (pups / dam) at 6 months but not 10 months of age. APAP also reduced gonocyte numbers, decreased mitotic activity, with a proposed mechanism of blocking DNA replication, and reduced stem cell proliferation at both the low and high dosage.

The authors conclude that APAP poses a significant risk to the reproductive health of all offspring regardless of sex.

Rossitto Jan. 2019; Rossitto Aug. 2019

A pair of studies on male[339] and female[340] offspring with *in utero* exposure to APAP (30mg/Kg) were performed by Rossitto et al. using CD-1 mice. The latter study used a combination of APAP with ibuprofen, but the former study examined APAP individually as well. The authors reported that exposure to APAP during the period gonadal development can lead to problems in male reproductive cell growth and development. This can result in delayed maturation of cells in the testes and a decrease in the number of sperm produced. They observed reduced sperm count and motility in adult males exposed to APAP *in utero*. These findings suggest that using APAP during this critical time may harm germ-line development, and these adverse effects could be passed on to future generations.

5. Other APAP Mouse Studies

Lee 2015

In a study by Lee et al. there was no reported toxicity on airway hyper-responsiveness (AHR) following APAP exposure during pregnancy or lactation.[341] It is important to note that this study by Lee et al. only dosed at 5mg/Kg, which is well below the therapeutic or toxic dose in mice. This dose selection likely stems from not converting the human mg/Kg dose to a HED in mice (see Table 1). For this example, a recommended 10mg/Kg human pediatric dosage produces a HED of 123mg/Kg for mice, so the tested 5mg/Kg would produce a HED of ~60mg/Kg in mice.

D. Rat Studies on APAP: Causality and Neurodevelopmental Toxicity

The following rat studies were reviewed in regard to general, reproductive, developmental, or neurodevelopmental toxicity of APAP in rats (Table 9). The strategy for identifying relevant studies is described in Appendix B.

Author	Year	Strain	Dose (mg/Kg)
Lubawy and Garrett	1977	Sprague-Dawley	0, 125, 250
Mancini et al.	1980	Sprague-Dawley	1200 - 2350 (LD50)

[339] Rossitto et al. Intergenerational effects on mouse sperm quality after in utero exposure to acetaminophen and ibuprofen. FASEB J. 2019 Jan;33(1):339-357. doi: 10.1096/fj.201800488RRR. Epub 2018 Jul 6. PMID: 29979629.

[340] Rossitto et al. In utero exposure to acetaminophen and ibuprofen leads to intergenerational accelerated reproductive aging in female mice. Commun Biol. 2019 Aug 13;2:310. doi: 10.1038/s42003-019-0552-x. PMID: 31428698; PMCID: PMC6692356.

[341] Lee et al. Perinatal paracetamol exposure in mice does not affect the development of allergic airways disease in early life. Thorax. 2015 Jun;70(6):528-36. doi: 10.1136/thoraxjnl-2014-205280. Epub 2015 Apr 3. PMID: 25841236; PMCID: PMC4453715.

Lin and Levy	1983	Lewis	0, 15, 300
Momma & Takeuchi	1983	Wistar	0, 10, 100, 1000
Harris et al	1988	Sprague-Dawley	n/a
Harris et al	1989	Sprague-Dawley	0, 250, 500µM
Stark et al.	1989	Sprague-Dawley	0, 250, 500µM
Stark et al.	1990	Sprague-Dawley	0,100,250,500,1000µM
Weeks et al.	1990	Sprague-Dawley	300µM
Gandy et al.	1990	Sprague-Dawley	0, 500, 1000, 1500
NTP	1993	F344/N	0, 80, 160, 310, 620, 1250
Ying & Lou	1993	Unspecified	0, 250, 500, 1000
Micheli et al.	1994	Wistar	0, 3000
Lister & McLean	1995	Unspecified	0, 125, 500
Lister & McLean	1997	Wistar	0, 60, 125, 500, 1000
Ratnasooriya & Jayakody	2000	Crossbred Albino	0, 500, 1000
Beck et al.	2001	Sprague-Dawley	0, 125, 250, 500
Ahmed et al.	2003	Sprague-Dawley	0, 500
Neto et al.	2004	Wistar	0, 125, 500, 1500
Oyagbemi et al.	2009	Wistar	0 or 3000
Posadas et al.	2010	Sprague-Dawley	0, 250, 500
Kristensen et al.	2011	Wistar	150, 250 and 350
Dean et al.	2012	Sprague Dawley	0, 40
Blecharz-Klin et al.	2013	Wistar	0, 10, 50
Oyedeji et al.	2013	Albino	0 & 7.5
Axelstad et al.	2014	Wistar	0, 350
Blazquez et al.	2014	Wistar	0, 400mg/Kg by prepregnant weight
Blecharz-Klin et al. (a & b)	2015	Wistar	0, 5, 15
Lichtensteiger et al.	2015	Wistar	0, 360
Blinova et al.	2016	Unspecified Albino	0, 500
Dean et al.	2016	Wistar	0, 350
Johansson et al.	2016	Wistar	0, 360
Blecharz-Klin et al.	2017	Wistar	0, 5, 15
Onaolapo et al.	2017	Wistar	0, 800
Axelstad et al.	2018	Wistar	0, 360
Blecharz-Klin et al.	2018	Wistar	0, 5, 15
Chen et al.	2018	Wistar	0, 50, 300
Hurtado-Gonzalez et al.	2018	Wistar	0, 350
Saeedan et al.	2018	Wistar	0, 50

Toyoda et al.	2018	F344 lean Zucker obese Zucker	0, 4.4, 13.8, 44.2, 142.1, 490.8 0, 4.7, 14.6, 46.5, 152.8, 521.9 0, 5.5, 17.3, 55.4, 169.2, 539.9
Blecharz-Klin et al.	2019	Wistar	0, 5, 15
Motawi et al.	2019	Wistar	0, 400
Vigo et al.	2019	Wistar	0, 100
Aleixo et al.	2020	Wistar	0, 350
Díaz-Estrada et al.	2020	Wistar	0, 100
Klein et al.	2020	Wistar	0, 350
Koehn et al.	2020	Sprague-Dawley	0, 7.5, 15, 30
Koyuncuoglu et al.	2020	Wistar	0, 3000
Lalert et al.	2020	Wistar	0, 200
Oksuz et al.	2020	Wistar	0, 20, 500
Pereira et al.	2020	Wistar	0, 350
Yilmaz et al.	2020	Wistar	0, 50, 125, 250, 500
Koehn et al.	2021	Sprague-Dawley	0, 7.5, 15, 30
Rigobello et al.	2021	Wistar	0, 35, 350
Suda et al.	2021	Sprague Dawley	0, 70.56
Taqa et al.	2021	Unspecified Albino	0, 50
Blecharz-Klin et al.	2022	Wistar	0, 5, 15
Chen et al.	2022	Sprague–Dawley	0, 100, 300
Herrington et al.	2022	Sprague Dawley	0, 51.97
Lalert et al.	2023	Sprague Dawley	0, 200

Table 9. Rat Studies Reviewed for General and Neurodevelopmental Toxicity. The human-rat conversion provided in Table 1 indicates a 1g dose in a 60Kg adult (16.6mg/Kg) when converted to a rat dose produces a HED of ~103mg/Kg. Based on ED_{50} data and HED calculations, a rat dose of ~100-150 mg/Kg is therapeutic, see Human Equivalent Dosages. Several studies report maternal and developmental impacts below this "therapeutic" HED. Oral dosages of 1000mg/Kg are reported to be tolerated, as described below. IP dosages ≥ 800mg have been reported to produce toxicity in rats, which varies by rat strain and animal age.

1. Studies examining whether exposure to acetaminophen caused behavioral changes.

Several studies investigated whether exposure to acetaminophen caused behavioral changes and/or associated biological changes in the brain.

Dean 2012

This study examined the effect of early postnatal APAP exposure on neuropathology and behavior.[342] As discussed above, APAP is a COX inhibitor that inhibits prostaglandins. During development, the cerebellum has a high expression of prostaglandin receptors. This study showed that inhibition of

[342] Dean et al. Prostaglandin E2 is an endogenous modulator of cerebellar development and complex behavior during a sensitive postnatal period. Eur J Neurosci. 2012 Apr;35(8):1218-29. doi: 10.1111/j.1460-9568.2012.08032.x. PMID: 22512254; PMCID: PMC3534986.

prostaglandins in rat pups during early postnatal life altered cerebellar Purkinje cell development, initially resulting in increased dendritic growth in both sexes. APAP was shown to increase spinophilin, which was used as a marker for dendritic spines in the study. APAP specifically increased spinophilin in the cerebellar cortex of pups treated from PN7 to PN13 at a dosage of 200ug (~40mg/Kg). This impact on dendritic spines resulted in later cerebellar atrophy in males only. In addition, social interaction and sensory threshold were altered in males. The dose of 40mg/Kg would produce an adult human equivalent dose (HED) of 6.5 mg/KG, which would be about 400 mg in a 60Kg adult. The authors report that this dosage,

> is the rectal dose of acetaminophen used in settings such as pediatric emergency rooms and intensive care units.

This study proposes that prostaglandins, produced by COX enzymes, are important for brain development, and especially in the cerebellum, which is involved in many different brain functions. The authors propose that there is an increasing awareness of cerebellar pathology in disorders such as ASD, and they suggest that the results from the study support the impact of the cerebellum, and thereby APAP, on neurodevelopmental disorders such as ASD.

Blecharz-Klin 2013, Blecharz-Klin 2017

In the 2013 study, three-month old male Wistar rats were given either 0, 10, or 50mg/kg/day of acetaminophen for eight weeks.[343] The modified Morris water maze task was used to measure impacts on behavioral performance and learning. There were no significant differences between the groups in the water maze test. During the second day of training, working memory improved in the 10 and 50mg/Kg dosages. The study also showed an increase in the mean swimming speed of treated animals. High Performance Liquid Chromatography (HPLC) was used to determine the brain concentrations of neurotransmitters and metabolites. There were significant differences in monoamines and metabolites between the experimental groups. The authors conclude that the experiment shows that eight weeks of treatment with APAP results in changes in behavior and working memory in rats. The acetaminophen dosages of 10mg/Kg and 50mg/Kg used in this study have HEDs of 1.6mg/Kg and 8mg/Kg, respectively. This would be about 100mg and 500mg for a 60Kg adult, which is 10-50% of a single extra-strength dose (1000mg).

In the 2017 study, the investigators examined the behavioral impacts on Wistar rats that resulted from exposure during pregnancy and lactation to APAP (0, 5, 15mg/kg).[344] Specifically, they examined spatial learning and memory consolidation and the level of physical and exploratory activity. They also performed analysis (by HPLC) on brain concentrations of monoamines, metabolites, and amino acids. They reported no difference in motor performance with APAP exposure, but the lower dose of APAP (5mg/Kg) improved spatial memory and altered exploratory behavior. They also reported a decrease in motor activity and head-dipping at this exposure, and an increase in the number of crossings in the Water Maze at this dose. There were also reported changes in the amino acid levels in the hippocampus and cortex caused by APAP exposure. The authors conclude that exposure to APAP during fetal or early postnatal life dose-

[343] Blecharz-Klin et al. Paracetamol--the outcome on neurotransmission and spatial learning in rats. Behav Brain Res. 2013 Sep 15;253:157-64. doi: 10.1016/j.bbr.2013.07.008. Epub 2013 Jul 11. PMID: 23850354.
[344] Blecharz-Klin et al. Paracetamol - Effect of early exposure on neurotransmission, spatial memory and motor performance in rats. Behav Brain Res. 2017 Apr 14;323:162-171. doi: 10.1016/j.bbr.2017.01.051. Epub 2017 Feb 3. PMID: 28163096.

dependently alters cognitive functions, spatial working memory, motor performance, and there were significant alterations of neurotransmission in rats. These low dose studies have resulted in a proposed model in which APAP improves memory and/or learning at low concentrations. Specifically, the dosages used in this study were a fraction of a daily therapeutic HED, with the tested range (5-15mg/Kg) producing HEDs of 0.8-2.4mg/Kg; this results in reference human (60Kg) dosages of 50-150mg, or only 5-15% of a single recommended 1000mg dosage.

Onaolapo 2017

This study examined whether APAP exposure produced injury in kidney, liver, and cortical neurons.[345] The experimental exposure was 800 mg/kg/day (IP) for three consecutive days. As indicated previously, this dosage and route of exposure is considered an APAP overdose, based on both HED (7.7g, which is greater than 7.5g, the dose identified as causing acetaminophen poisoning in humans) and experimental findings in this and other rat studies. At this dosage, the authors report significantly reduced body weight, reduced locomotion, increased grooming, decreased spatial memory, and increased anxiety, with significant changes in clinical chemistry, including elevated AST, ALT, urea, and creatinine.

Blecharz-Klin 2018

This study investigated the effect of perinatal exposure to APAP on social behavior and levels of brain-derived neurotrophic factor (BDNF) in rats.[346] Wistar rats were exposed *in utero* during pregnancy and early life to low doses of APAP (0, 5, 15mg/kg). Animals exposed to APAP exhibited a lower total frequency of social interactions and greater pinning behavior, and rats from the 15mg/kg group exhibited a greater interest in objects in the novel object recognition tests. The striatum of rats exposed to APAP had a two-fold decrease in levels of BDNF in the striatum portion of the brain. The striatum is known to be involved in the integration of social information, cognitive processes and reward coding in the brain. And BDNF promotes the maturation, survival, and function of neurons.

Saeedan 2018

To examine the interactions between fever and APAP treatment and ASD risk, these investigators developed an experimental rat model of vaccine-induced fever with subsequent treatment for fever reduction by APAP.[347] This study is significant because it addresses the relative risk of fever and APAP to produce ASD, or neurodevelopmental toxicity. This is commonly referred to as confounding by indication in epidemiology studies, where a medical treatment may be associated with causing damage, but the damage can actually be caused by the indication or underlying illness being treated.

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[345] Onaolapo et al. l-Methionine and silymarin: A comparison of prophylactic protective capabilities in acetaminophen-induced injuries of the liver, kidney and cerebral cortex. Biomed Pharmacother. 2017 Jan;85:323-333. doi: 10.1016/j.biopha.2016.11.033. Epub 2016 Nov 23. PMID: 27889232.

[346] Blecharz-Klin et al, Early paracetamol exposure decreases brain-derived neurotrophic factor (BDNF) in striatum and affects social behaviour and exploration in rats, Pharmacology, Biochemistry and Behavior 168 (2018) 25–32, DOI:/10.1016 /j.pbb.2018.03.044

[347] Saeedan et al. Effect of early natal supplementation of paracetamol on attenuation of exotoxin/endotoxin induced pyrexia and precipitation of autistic like features in albino rats. Inflammopharmacology. 2018 Aug;26(4):951-961. doi: 10.1007/s10787-017-0440-2. Epub 2018 Jan 11. PMID: 29327281.

In this study, pups were treated with measles mumps rubella (MMR) vaccine, diphtheria tetanus and pertussis (DPT) vaccines, or lipopolysaccharide (LPS), individually and with subsequent APAP treatment at 50 mg/kg. Control and treated pups were evaluated for growth and development. The authors report a correlation between social-behavioral testing results and inflammatory markers in the pups. They report that all treatment groups showed significant alteration in the behavioral changes and increased oxidative markers (TBARS) and inflammatory markers. Exposure to APAP (50 mg/kg sc.) on PND5 resulted in the highest increase in TBARS in examined brain tissue. APAP was also reported to produce significant and time dependent impacts on eye opening, locomotor activity, olfactory discrimination, and on entries in the elevated plus maze. Based on the results that all exposures resulted in increased pathology and increased oxidative stress, the authors propose that both the indication (fever) and treatment (APAP) are causative. Specifically, they propose that dysregulated immune/inflammatory circuits and/or increased catecholamine in the brain (due to sulfur and/or amine disruptions induced by APAP) can increase neurobehavioral and neurotoxic effects in newborn brains with increased underlying susceptibility.

Klein 2020

This study examined whether APAP caused behavioral changes in Wistar rats exposed *in utero* to APAP (350mg/Kg).[348] Treatments were via gavage (350 mg/kg/day) or water from GD6 until delivery. The general toxicity endpoints included body weight and food intake. Behavioral evaluation occurred on PND10 (nest seeking test), PND27 (behavioral stereotypy), PND28 (three chamber sociability test and open field) and PND29 (hot plate and elevated plus-maze). Reduced glutathione (GSH) and brain derived neurotrophic factor (BDNF) levels were also quantified in the prefrontal cortex and hippocampus on PND22. Exposure to APAP impaired nest seeking behavior and decreased rostral grooming in the elevated plus maze. Alterations in glutathione levels or BDNF were not observed in the prefrontal cortex or hippocampus. Behaviors on the hot plate and sociability tests were unaffected by APAP. The authors conclude that the data support APAP as a neurotoxicant.

Rigobello 2021

Rigobello et al.[349] proposed to examine if perinatal exposure to APAP, at human-relevant doses, could alter the brain and behavior of offspring. Wistar dams were gavaged daily with water or APAP (35 mg/kg or 350 mg/kg) from GD6-PND21. Behavior testing occurred at PND10 (nest seeking test), PND27 (behavioral stereotypy), and PND28 (three-chamber sociability test and open field). Compared to control animals, perinatal exposed males had augmented stereotyped behavior and ambulation in an open-field test. They also reported a reduction in exploratory behavior in the three-chamber sociability test (high dose). APAP decreased the levels of glutathione (low dose, hippocampus). The authors propose that use of APAP during pregnancy and breastfeeding is a potential risk factor for neurological disorders.

Suda 2021

[348] Klein et al. Gestational exposure to paracetamol in rats induces neurofunctional alterations in the progeny. Neurotoxicol Teratol. 2020 Jan-Feb;77:106838. doi: 10.1016/j.ntt.2019.106838. Epub 2019 Oct 20. PMID: 31644948.
[349] Rigobello et al. Perinatal exposure to paracetamol: Dose and sex-dependent effects in behaviour and brain's oxidative stress markers in progeny. Behav Brain Res. 2021 Jun 25;408:113294. doi: 10.1016/j.bbr.2021.113294. Epub 2021 Apr 6. PMID: 33836167.

These study authors exposed pregnant rats to APAP (14.7mg/Kg) from PND4-10.[350] This dose was described as a pediatric human dosage (mg/Kg) but was not converted to a HED. The antioxidants cysteine and mannitol were included to prevent accumulation of NAPQI. Western diet, ampicillin (15 mg/Kg), and killed bacteria (1E6) with antibiotics were also examined as potential stressors. The antioxidants were reported to not correct the impact of APAP on behavior. Treatment with acetaminophen was associated with a significant increase in rearing, an asocial behavior, regardless of whether the animals were exposed to other oxidative stress factors or antioxidants. The authors suggest,

> …the inclusion of multiple oxidative stress inducers in the model did not significantly alter the outcome, suggesting that the neurodevelopmental effects we observed are, at least to some extent, independent of oxidative stress.

Measurements of oxidative stress, glutathione, or products produced by NAPQI were not determined in this study. The study authors report: "it is apparent that the currently approved use of acetaminophen in infants and children would never have passed pre-clinical testing if the drug were evaluated based on current standards." And the authors conclude:

> Given current evidence and the fact that the primary target organ for the drug is the brain, it is now quite apparent that proof of absence of damage to the liver should never have been considered adequate proof of safety.

Díaz-Estrada 2021

Based on the endocrine and neuroendocrine impacts of APAP on the developing rat brain, a study was performed to examine the impact on sexual interactions following *in utero* APAP treatments.[351] This study addressed what was termed the sexual partner preference (SPP) of adult male rats treated with APAP. The authors hypothesized that inhibition of cyclooxygenase by APAP during brain development would result in increased homosexual SPP. Treatment with APAP was from GD16-20 of pregnancy, with pregnant female rats receiving either saline or APAP (50mg/Kg, SC) every 12 h. On PND60, half of the male offspring were exposed to sexual experience with receptive females during 5 trials, while the other half remained sexually naive. On PND90, all male offspring were tested for SPP with one sexually receptive female and one stud male. The authors report that APAP-experienced males displayed SPP for females, but APAP-naive males failed to display heterosexual SPP (Figure 28). The authors conclude that APAP disrupted neurotypical heterosexual behavior in rats (nature mechanisms), but early heterosexual experiences restored heterosexual SPP (nurture mechanisms). The dose tested in this study produces a HED of ~8mg/Kg, or about 500mg in a 60Kg adult, or half the recommended therapeutic dose of 1000mg.

[350] Suda et al. Therapeutic doses of acetaminophen with co-administration of cysteine and mannitol during early development result in long term behavioral changes in laboratory rats. PLoS One. 2021 Jun 25;16(6):e0253543. doi: 10.1371/journal.pone.0253543. PMID: 34170958; PMCID: PMC8232535.

[351] Díaz-Estrada et al. Nature and nurture of sexual partner preference: Teachings from prenatal administration of acetaminophen in male rats. Horm Behav. 2020 Aug;124:104775. doi: 10.1016/j.yhbeh.2020.104775. Epub 2020 Jun 6. PMID: 32422195.

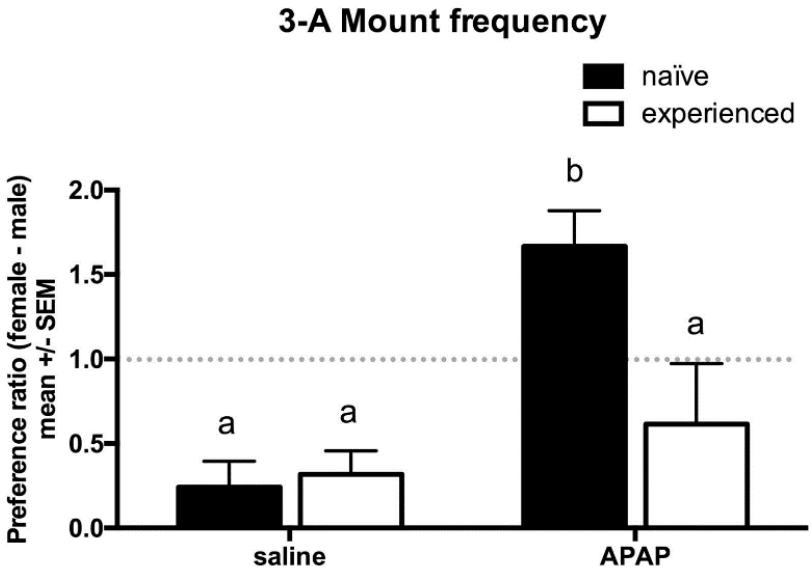

Figure 28. Preference Ratio of Copulatory Sexual Behaviors. Ratios of 1.0 indicate no preference, whereas deviation to 2 indicatesmale preference, and deviation to 0 indicate female preference. Different letters indicate significant findings ($p < .05$) between groups. Naïve in utero exposed APAP males show increased homosexual mounting.

2. Studies examining whether exposure to acetaminophen caused biological changes in the brain.

Several studies investigated whether exposure to acetaminophen caused biological changes in the brain. (As noted above, some of the studies in section 1 also examined biological changes in the brain.)

Harris 1988, Harris 1989

Harris in 1988 used the rat embryo culture model to show that glutathione (GSH) was a modifier of teratogenesis for diverse chemicals, including valproic acid (VPA).[352] The interaction between VPA and glutathione (GSH) is of particular interest because VPA exposure has been recognized as causing behaviors representing ASD, and VPA-exposed rodents are regularly used to model ASD. These researchers then examined the impact of APAP on neurulation staged rat embryos in culture.[353] Briefly, the addition of APAP (250 or 500μM) resulted in abnormal closure of the anterior neuropore of cultured rat embryos. Failure to close the anterior neuropore results in either exencephaly or anencephaly, both of which are neural tube defects (NTDs).

Micheli 1993

[352] Harris et al. Glutathione status and the incidence of neural tube defects elicited by direct acting teratogens in vitro. Teratology. 1988 Jun;37(6):577-90. doi: 10.1002/tera.1420370607. PMID: 3135633.
[353] Harris et al. Abnormal neurulation induced by 7-hydroxy-2-acetylaminofluorene and acetaminophen: evidence for catechol metabolites as proximate dysmorphogens. Toxicol Appl Pharmacol. 1989 Dec;101(3):43
2-46. doi: 10.1016/0041-008x(89)90192-0. PMID: 2603160.

This study examined the effect of acetaminophen on glutathione levels in the brain.[354] Male albino Wistar rats were treated with APAP. One group (n=9) was given 3 gm/kg of APAP while the control group (n=8) received only the vehicle (2% gum arabic in saline). The rats were decapitated 6 hours after administration to avoid the influence of circadian rhythm on GSH levels. This time limit was chosen because peak concentrations of APAP in rat brains occur approximately 6 hours after dosing. APAP and GSH concentrations were determined in the following areas: hypothalamus, corpus striatum, hippocampus, limbic area, pons, medulla oblongata, cerebral cortex, and cerebellum. The authors reported that large reductions in GSH levels were seen in the hypothalamus (- 20%), medulla oblongata (- 14%), and cerebral cortex (- 24%). They also propose that a 20% to 30% decrease of GSH levels is considered significant and able to result in cell injury or cell death if GSH is not restored.

Posadas 2010

This study examined whether exposure to APAP caused neurotoxic effects in rats.[355] The study authors report:

> The data presented here establish, for the first time, a direct neurotoxic action by APAP both in vivo and in vitro in rats at doses below those required to produce hepatotoxicity and suggest that this neurotoxicity might be involved in the general toxic syndrome observed during patient APAP overdose...

Experimentally, the study used both *in vitro* culture conditions and *in vivo* treatment of animals. The impact of 250 and 500mg/Kg APAP in rats was reported to result in concentrations from 1-2mM in plasma and 1-3mM in cerebral spinal fluid. These concentrations were also reported to be neurotoxic both *in vitro* and *in vivo* (Figure 29). The results of acute treatment with APAP were shown to produce apoptosis and DNA damage in the brains of exposed animals.

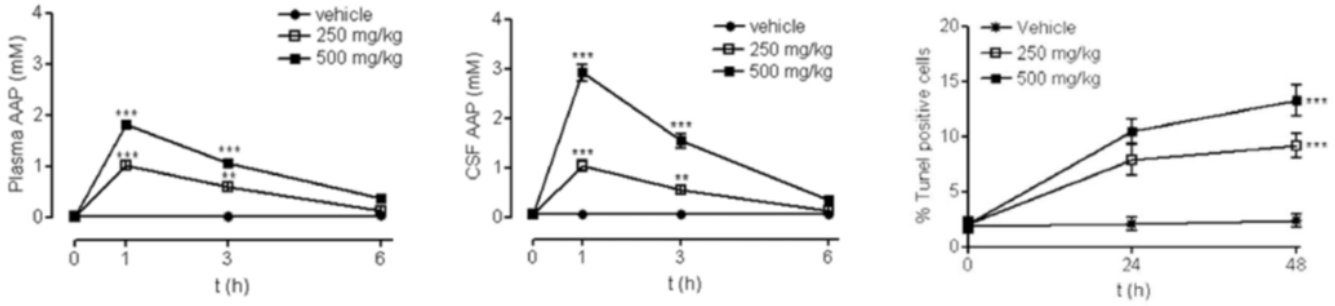

Figure 29. Acetaminophen (AAP) Dosing and Plasma and Cerebral Spinal Fluid Concentrations. Dosages of 250 or 500mg/Kg were administered to rats. The resulting concentrations of AAP/APAP are in the mM range for both (Left) plasma and (Middle) cerebral spinal fluid (CSF). (Right) Tunel positive cells are undergoing apoptosis, with increased DNA fragmentation in animals dosed at 250 and 500mg/Kg.[356]

[354] Micheli et al. Effect of acetaminophen on glutathione levels in several regions of the rat brain. Curr Ther Res 1993;53:730–6. https://doi.org/10.1016/S0011-393X(05)80745-3

[355] Posadas et al. Acetaminophen induces apoptosis in rat cortical neurons. PLoS One. 2010 Dec 10;5(12):e15360. doi: 10.1371/journal.pone.0015360. PMID: 21170329; PMCID: PMC3000821.

[356] Posadas et al. Acetaminophen induces apoptosis in rat cortical neurons. PLoS One. 2010 Dec 10;5(12):e15360. doi: 10.1371/journal.pone.0015360. PMID: 21170329; PMCID: PMC3000821.

Blecharz-Klin 2014

This study examined whether exposure to APAP caused effects on concentrations of amino acids in the prefrontal cortex, hippocampus, hypothalamus, and striatum of Wistar rats, as measured using High Performance Liquid Chromatography.[357] The study authors report a decrease in the concentration of amino acids in the striatum (glutamine, glutamic acid, taurine, alanine, aspartic acid) and hypothalamus (glycine) between APAP treated rats compared to controls. In the prefrontal cortex, APAP also resulted in increased levels of γ-aminobutyric acid (GABA). The authors conclude that long-term treatment with APAP has a significant effect on the levels of amino acids in the striatum, hypothalamus, and prefrontal cortex of rats.

Blecharz-Klin 2015a, 2015b

These study authors examined whether early life administration of APAP would affect the levels of neurotransmitters in the spinal cord of rat pups.[358] The Wistar rats were exposed to APAP at 0, 5, or 15mg/Kg per day prenatally and for two months postnatal. High Performance Liquid Chromatography was used to determine the level of monoamines, metabolites, and amino acids in the spine of rats. The study authors reported that during this critical period of neurodevelopment, the content of aspartic and glutamic acids in the spine were increased by APAP. A similar study by this group also indicated that APAP also alters the dopaminergic system in the medulla oblongata.[359] The acetaminophen dosages of 5mg/Kg and 15mg/Kg used in this study have HEDs of 0.8mg/Kg and 2.4mg/Kg, respectively. This would be about 50mg and 150mg for a 60Kg adult, which is between 5% and 15% of single extra-strength dose (1000mg).

Lichtensteiger 2015

This research group examined the impact of APAP on Wistar rats during sexual brain differentiation. Specifically, gene expression was analyzed by real-time RT-PCR in the preoptic area and ventromedial hypothalamus animals exposed to APAP or various mixtures of endocrine disrupting chemicals (EDCs).[360] This included an androgen mix (A-mix) and estrogen mix (E-mix) and a combination mixture (A+E+APAP = AEP-mix). Sex and region-specific gene expression patterns in brains from offspring were reported. The A or E-mix was administered by oral gavage to rat dams from gestation day 7 until weaning. The AEP-mix or APAP alone was administered from GD13–GD19. The authors conclude that APAP produces sex and region-specific gene expression patterns in developing rat brain during a critical, sex hormone-sensitive period. The dosage used in these series of studies (350mg/Kg) produces a HED of ~56.5mg/Kg (3.4g in a 60 Kg adult), which is 3.4X a single 1000mg human therapeutic dose, and somewhat less than the recommended limit of 4000 mg in 24 hours.

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[357] Blecharz-Klin et al. Paracetamol impairs the profile of amino acids in the rat brain. Environ Toxicol Pharmacol. 2014 Jan;37(1):95-102. doi: 10.1016/j.etap.2013.11.004. Epub 2013 Nov 13. PMID: 24316461.

[358] Blecharz-Klin et al. Effect of prenatal and early life paracetamol exposure on the level of neurotransmitters in rats--Focus on the spinal cord. Int J Dev Neurosci. 2015 Dec;47(Pt B):133-9. doi: 10.1016/j.ijdevneu.2015.09.002. Epub 2015 Sep 25. PMID: 26390956.

[359] Blecharz-Klin et al. Developmental exposure to paracetamol causes biochemical alterations in medulla oblongata. Environ Toxicol Pharmacol. 2015 Sep;40(2):369-74. doi: 10.1016/j.etap.2015.07.001. Epub 2015 Jul 4. PMID: 26233562.

[360] Lichtensteiger et al. Differential gene expression patterns in developing sexually dimorphic rat brain regions exposed to antiandrogenic, estrogenic, or complex endocrine disruptor mixtures: glutamatergic synapses as target. Endocrinology. 2015 Apr;156(4):1477-93. doi: 10.1210/en.2014-1504. Epub 2015 Jan 21. PMID: 25607892.

Blecharz-Klin 2019

This study examined the interaction of perinatal exposure to APAP with the hypothalamus.[361] The interest in hypothalamic changes were reported to stem from the influence of APAP on fever, which is influenced by this region of the brain. Wistar rats were exposed during pregnancy and lactation to low doses of APAP (0, 5, 15mg/kg). The investigators measured the resulting levels of monoamines, metabolites, and amino acids in the hypothalamus of offspring. The concentration of noradrenaline in the hypothalamus was found to be different between groups. Dopamine and homovanillic acid were increased in the hypothalamus of animals treated at 5mg/Kg. There was also a significant difference in the concentration of glutamic acid at this dose. The authors conclude that perinatal exposure to APAP alters neurotransmitters and metabolites in the brain; specifically, dopaminergic, noradrenergic, and glutamic acid in the hypothalamus. They also propose that this study defines one of the mechanisms by which APAP can interfere with normal reproductive-testicular function, i.e., disturbance of the catecholamine-dependent hypothalamus-testicular pathway.

Motawi 2019

This study examined biological changes in the brain caused by exposure to APAP at therapeutically relevant dosages.[362] In this study, male Wistar albino rats were treated with APAP (400 mg/kg, via gavage) for 28 days. Results indicated significant alteration in brain neurotransmitters and thyroid-hormones, including decreased thyroid releasing hormone (TRH), thyroid stimulating hormone (TSH), triiodothyronine (T3), and thyroxine (T4). There were also significant changes in oxidative stress markers in the brain, including significant decreases in GSH. These changes coincided with increased DNA fragmentation (Comet assay) in brain tissue, with associated pathologies, including edema, hyalinized axon, and an increased number of inflammatory cells between neurons. The tested dosage is therapeutically relevant. A 400mg/Kg rat dosage would be equivalent to a dose of ~3.9g in a 60Kg human adult, which is below the maximum recommended dosage of 4 g per 24 hours. The temporality of these events needs additional study, such as when the pathology begins to develop, when biomarkers first begin to deviate from controls, and how long recovery takes after exposure to APAP as a function of acute versus chronic durations of exposure. Nevertheless, the results from this study indicate that given the described timing, dose, and duration of exposure, APAP causes oxidative stress, DNA damage, and brain pathologies.

Koehn 2020

This research examined whether *in utero* APAP exposure caused changes in gene expression and whether it had an effect on permeability of the blood-brain barrier.[363] The investigators administered APAP (IP) twice daily to Sprague-Dawley rats from embryonic day E15 to E19 (chronic) or as a single dose at E19 (acute) at 3.75mg/Kg or 15mg/Kg. The authors comment that "[t]he doses used were within the clinically

[361] Blecharz-Klin et al. Hypothalamus - Response to early paracetamol exposure in male rats offspring. Int J Dev Neurosci. 2019 Aug;76:1-5. doi: 10.1016/j.ijdevneu.2019.05.004. Epub 2019 May 21. PMID: 31125683.
[362] Motawi et al. Protective effects of betanin against paracetamol and diclofenac induced neurotoxicity and endocrine disruption in rats. Biomarkers. 2019 Nov;24(7):645-651. doi: 10.1080/1354750X.2019.1642958. Epub 2019 Aug 5. PMID: 31305161.
[363] Koehn et al. Effects of paracetamol (acetaminophen) on gene expression and permeability properties of the rat placenta and fetal brain. F1000Res. 2020 Jun 8;9:573. doi: 10.12688/f1000research.24119.2. PMID: 32934805; PMCID: PMC7477648.

recommended range (0.5g to 4g in 24 hours in adults)," but the investigators omitted rat-human dose conversion. The doses of 3.75mg/Kg and 15mg/Kg correspond to a HED of about 37.5mg and 150mg in a 60Kg adult, which is well below the therapeutic dose.

On GD19, dams were treated, followed by surgical collection of fetal brains. Fetal and maternal plasma and cerebrospinal fluid were collected and tested for a-fetoprotein and interleukin 1β (IL1β). Labeled 14C-sucrose was used to examine placental permeability. The results showed down-regulation of three acute phase plasma proteins (α-fetoprotein, transferrin, transthyretin) with acute and chronic treatments and up-regulation of immune-response related genes, particularly cytokines, with the chronic treatment. The amount of IL1β in the blood of treated fetuses increased, but no IL1β was found in the controls or in the maternal blood of APAP treated animals. The authors comment that their data "confirms that paracetamol was indeed eliciting an inflammatory response but only on the fetal side of the placental circulation." [364] There was no evidence of increased blood-brain barrier permeability in the fetal brain. The authors suggest that APAP can induce an immune-inflammatory-like response in the placenta if used during pregnancy.

3. Studies Examining Acetaminophen, Glutathione Depletion, and Oxidative Stress

Rat studies were conducted to examine the impact of APAP on glutathione levels and toxicity from oxidative stress.

Stark (1989)

This study showed that the depletion of GSH in cultured rat embryos potentiates the developmental toxicity of APAP.[365] Metabolites of APAP, including NAPQI were also shown to cause disruption of neural tube closure and to decrease viability of embryos.

Stark (1990)

This study characterized APAP in relation to NAPQI and various APAP-analogs using rat embryo culture.[366] Direct additions of APAP to the cultured embryos produced an increased incidence of neural tube defects (NTDs), open anterior neuropores. This contrasted with intra-amniotic microinjections of APAP, which failed to produce NTDs. The APAP metabolite NAPQI produced NTDs and reduced viability when added to the culture directly or via intra-amniotic microinjections. The authors conclude that the metabolic conversion of APAP by the yolk sac (the tissue surrounding the embryo) was associated with NTDs.

Weeks (1990)

[364] Koehn et al. Effects of paracetamol (acetaminophen) on gene expression and permeability properties of the rat placenta and fetal brain. F1000Res. 2020 Jun 8;9:573, p.24. doi: 10.12688/f1000research.24119.2. PMID: 32934805; PMCID: PMC7477648.
[365] Stark et al. Influence of electrophilic character and glutathione depletion on chemical dysmorphogenesis in cultured rat embryos. Biochem Pharmacol. 1989 Aug 15;38(16):2685-92. doi: 10.1016/0006-2952(89)90555-8. PMID: 2764988.
[366] Stark et al. Dysmorphogenesis elicited by microinjected acetaminophen analogs and metabolites in rat embryos cultured in vitro. J Pharmacol Exp Ther. 1990 Oct;255(1):74-82. PMID: 2213573.

This study confirmed the developmental toxicity of APAP (300μM) on rat embryos.[367] The study examined APAP and APAP metabolites by orally treating rats (1g/Kg) and monkeys (400mg/Kg) with APAP and collecting serum from animals for subsequent testing and rat embryo culture. The authors reported that APAP reduced the levels of glutathione (GSH) in the yolk sac (the tissue surrounding the embryo), and that APAP toxicity was potentiated by depletion of GSH. They also demonstrated that the addition of the glutathione precursor N-acetylcysteine (NAC) would rescue the embryos from the harmful effects of APAP. The resulting developmental toxicities from treated rats and monkeys demonstrated time dependent and concentration dependent interactions, with 70 minutes in the monkey serum, and 15 and 30 minutes in the rat serum showing the most toxicity to exposed rat embryos in culture. This study demonstrated dose-responsive developmental toxicity of APAP and metabolites thereof on cultured rat embryos (Table 10).

Treatment	Acetaminophen concentrations in culture (μM)	No. of embryos (abnormal/total)	Number of embryos: Open neutral tubes	Compressed optic vesicles	Incomplete body curvature	Embryo protein (μg/embryo ± SEM)
Acetaminophen						
	0	0/6	0	0	0	218 ± 11
	300	5/10	4	4	5	195 ± 8
	450	12/12	8	10	8	109 ± 7
	600	10/10	6	9	8	65 ± 5
	750	7/7	6	7	6	42 ± 4

Table 10. Teratogenic Effects of Acetaminophen on Cultured Rat Embryos.[367]

Gandy (1990)

This study examined several compounds for the ability to perturb glutathione (GSH) levels in the testes and epididymides as well as liver following single acute dosages to rats.[368] The authors propose a protective role for GSH in reproductive tissues, and that GSH detoxification may be critical in preventing chemical-induced germ-cell mutations. Moreover, the study indicates that the GSH detoxication system is present in male reproductive tissue, including GSH, GSH S-transferases, GSH peroxidase, GSH reductase, and gamma-glutamyl transpeptidase. The authors report that APAP (1500 mg/kg, IP) caused a decrease in hepatic GSH to 31% of control levels at 1hr post treatment, and testicular GSH was significantly depleted to 68% of controls values at 1hr. Also, while liver GSH declined from 4-18hrs, there was a recovery of testicular GSH by 2hrs posttreatment. They authors propose that lowering GSH in the male reproductive tract may be a way that chemicals can cause mutations in sperm cells.

Micheli (1994)

[367] Weeks et al. Acetaminophen toxicity to cultured rat embryos. Teratog Carcinog Mutagen. 1990;10(5):361-71. doi: 10.1002/tcm.1770100502. PMID: 1981948.

[368] Gandy et al. Effects of selected chemicals on the glutathione status in the male reproductive system of rats. J Toxicol Environ Health. 1990;29(1):45-57. doi: 10.1080/15287399009531370. PMID: 2299686.

This study examined the impact of APAP on testicular glutathione (GSH) levels.[369] This study was performed in Wistar rats and the authors report that APAP reached a peak concentration (Cmax) of 114ug/g at 6 hours post oral administration of 3g/Kg. Testicular GSH was also shown to significantly decrease to approximately 75% of controls at 4 hours post treatment. The authors propose that APAP and NAPQI exposure can produce specific toxicities in the organs and tissues exposed to these chemicals and can potentiate the toxicity of other toxic agents by reducing GSH in those tissues.

Acharya 2010

This study demonstrated that while APAP depletes glutathione, treatment with NAC, hypotaurine (HYTAU), or taurine (TAU) mitigate glutathione loss in both plasma and liver.[370]

Toyoda 2018.

This study examined genetic differences and obesity as a potential modifier of APAP-toxicity.[371] Male F344 and Zucker (lean and obese) rats were administered 0, 80, 253, 800, 2,530, or 8,000 ppm APAP in the diet for 13 weeks. There was no significant toxicity related to APAP treatment in all three strains. The body weight gain in F344 and lean Zucker rats was reduced. Other notable changes at the high dose include increased brain and liver weight in the F344 rats, and increased liver weight with decreased testicular weight in the lean Zucker rats. The results suggested that Zucker rats may be less susceptible to APAP toxicity than lean rats. As indicated below, see NTP, a 6,000ppm APAP diet results in an exposure of approximately 300mg/Kg/day, so the high dosage (8,000ppm) is expected to result in an exposure of ~400mg/Kg/day. This dosage produced no overt liver toxicity in any of the rat strains examined. The high dosage (8,000 ppm or 400mg/Kg/day), results in a HED of ~65mg/Kg or ~3.9g/day in a 60Kg human.

Koyuncuoğlu 2020

This study examined whether males have greater propensity for toxicity from APAP than females.[372] The investigators used APAP overdosing (3g/Kg, IP) in male rats and female rats that underwent ovariectomy (OVX) or sham-OVX. Results indicated the hepatotoxicity of APAP was less severe in female rats, but renal toxicity was not influenced by sex or by the lack of ovarian hormones. The authors also reported that pretreatment with estrogen or ER agonists provided protective effects against APAP-induced hepato- and renal toxicity. The antioxidant activity of estrogens was thereby supported to mitigate these and potentially other toxicities produced by APAP.

Herrington (2022)

[369] Micheli et al. Effect of acetaminophen on glutathione levels in rat testis and lung. Environ Health Perspect. 1994 Nov;102 Suppl 9(Suppl 9):63-4. doi: 10.1289/ehp.94102s963. PMID: 7698087; PMCID: PMC1566779.
[370] Acharya and Lau-Cam. Comparison of the protective actions of N-acetylcysteine, hypotaurine and taurine against acetaminophen-induced hepatotoxicity in the rat. J Biomed Sci. 2010 Aug 24;17 Suppl 1(Suppl 1):S35. doi: 10.1186/1423-0127-17-S1-S35. PMID: 20804611; PMCID: PMC2994383.
[371] Toyoda et al. A 13-week subchronic toxicity study of acetaminophen using an obese rat model. J Toxicol Sci. 2018;43(7):423-433. doi: 10.2131/jts.43.423. PMID: 29973474.
[372] Koyuncuoğlu et al. Estrogen receptor agonists protect against acetaminophen-induced hepatorenal toxicity in rats. Life Sci. 2020 Dec 15;263:118561. doi: 10.1016/j.lfs.2020.118561. Epub 2020 Oct 10. PMID: 33045213.

A study by Herrington et al., hypothesized that exposure to ghrelin prior to exposure to APAP would mitigate the behavioral effects of APAP exposure.[373] Ghrelin is a neuroactive peptide commonly described as a signaling molecule for hunger, but it is also reported to processes antioxidant properties. The authors report that ghrelin can restore altered anxiety-associated behaviors in rats. They conclude that the results are the first to demonstrate that ghrelin can mitigate the effects of perinatal APAP exposure in rats. This study is compelling because ghrelin was given after APAP exposure, so ghrelin may have potential therapeutic benefits similar to providing NAC to patients or animals overdosed with APAP. Ghrelin is also reported to modify serotonin,[374] and influence appetite by modifying cannabin[375]signaling.[376] These various interactions indicate that the ability of ghrelin to rescue disrupted neurodevelopment caused by APAP may be due to modified oxidative stress and convergent signaling interactions between APAP and ghrelin.

4. Studies Examining Acetaminophen and Biological Changes in Reproductive or Other Tissues.

In addition to the rat studies described above that examined biological changes to the brain, rat studies were used to examine whether APAP caused biological changes in reproductive or other tissues.

Momma 1983

This study examined numerous chemicals and medications, including APAP, for impacts on fetal ductal constriction.[377] Wistar rat dams were exposed to APAP at doses of 10, 100, or 1000 mg/Kg. Transverse sections of the main pulmonary artery (PA) and the ductus arteriosus (DA) were examined, and measured diameters were used calculate a DA/PA ratio for each chemical (Figure 30). APAP at higher doses induced ductal constriction. This impact of APAP on the ductus arteriosus is consistent with clinical studies and was summarized in recent reviews on the treatment or closure of patent ductus arteriosus (PDA) in infants.[378] This activity is proposed to be due to inhibition of cyclooxygenases.

[373] Herrington et al. Elevated ghrelin alters the behavioral effects of perinatal acetaminophen exposure in rats. Dev Psychobiol. 2022 Mar;64(3):e22252. doi: 10.1002/dev.22252. PMID: 35312061.
[374] Brunetti et al. Effects of ghrelin and amylin on dopamine, norepinephrine and serotonin release in the hypothalamus. Eur J Pharmacol. 2002 Nov 15;454(2-3):189-92. doi: 10.1016/s0014-2999(02)02552-9. PMID: 12421646.
[375] Burdyga et al. Ghrelin receptors in rat and human nodose ganglia: putative role in regulating CB-1 and MCH receptor abundance. Am J Physiol Gastrointest Liver Physiol. 2006 Jun;290(6):G1289-97. doi: 10.1152/ajpgi.00543.2005. Epub 2006 Jan 19. PMID: 16423919.
[376] Fride et al. Endocannabinoids and food intake: newborn suckling and appetite regulation in adulthood. Exp Biol Med (Maywood). 2005 Apr;230(4):225-34. doi: 10.1177/153537020523000401. PMID: 15792943.
[377] Momma and Takeuchi. Constriction of fetal ductus arteriosus by non-steroidal anti-inflammatory drugs. Prostaglandins. 1983 Oct;26(4):631-43. doi: 10.1016/0090-6980(83)90200-9. PMID: 6658007.
[378] Jasani et al. Paracetamol (acetaminophen) for patent ductus arteriosus in preterm or low birth weight infants. Cochrane Database Syst Rev. 2022 Dec 15;12:CD010061. doi: 10.1002/14651858.CD010061.pub5. PMID: 36519620.

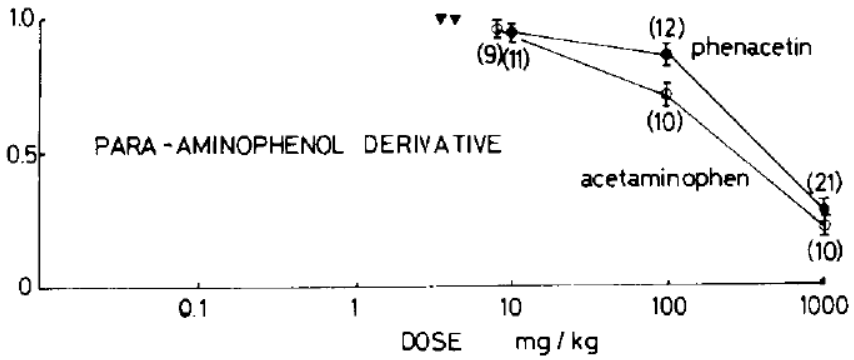


Figure 30. Fetal Ductal Constriction Caused by Acetaminophen. The main pulmonary artery (PA) and the ductus arteriosus (DA) inner diameters were measured (DA/PA ratio) for APAP and phenacetin. APAP induces ductal constriction, as indicated by a decreasing DA/PA ratio at increasing dosages of APAP.

NTP 1993

The NTP performed studies on mice and rats.[379] These studies were conducted by administering APAP in feed to F344/N rats of each sex for 14 days, 13 weeks, and 2 years. The 13-week exposure study reports,

> Acetaminophen-related lesions were observed in the liver (necrosis, chronic active inflammation, hepato- cytomegaly), kidney (tubule cast, tubule necrosis, tubule regeneration), reproductive organs (atrophy of testis, ovary, and uterus), thymus and lymph nodes (lymphoid depletion) of rats that received 25,000 ppm, and of the liver (chronic active inflammation, hepatocytomegaly) and testis (atrophy) of male rats receiving 12,500 ppm.

The NTP reports testicular atrophy at 6200, 12500, and 25000, and these results are summarized as follows,

> Testicular atrophy was observed in all male rats that received 25,000 ppm and in one male from each of the 6,200 and 12,500ppm groups. In the most severe cases, the lesion consisted of nearly complete loss of the germinal epithelium and was usually associated with complete absence of sperm in the epididymis. Atrophy of the uterus and ovary was observed in female rats that received 25,000ppm. The atrophic ovaries were small and had fewer follicles and corpora lutea, while atrophic uteri appeared small on gross observation.

Based on food consumption, the NTP reports that 6000ppm results in an exposure of approximately 300mg/Kg/day, so the foods formulated with 12,500ppm and 25,000ppm result in exposures of ~625mg/Kg/day and ~1250mg/Kg/day, respectively. The HEDs (Table 1) for these dosages (300, 625, 1250mg/Kg) are ~50mg/Kg/day, ~100mg/Kg/day, and ~200mg/Kg/day, making reference human (60Kg) exposures of 3g/day, 6g/day, and 12g/day, respectively.

Lister 1995, Lister 1997

[379] National Toxicology Program. NTP Toxicology and Carcinogenesis Studies of Acetaminophen (CAS No. 103-90-2) in F344 Rats and B6C3F1 Mice (Feed Studies). Natl Toxicol Program Tech Rep Ser. 1993 Jan;394:1-274. PMID: 12637965.

This pair of studies investigated the effects of acetaminophen on DNA synthesis.[380,381] The first of these studies used dosages of 0, 125, 500mg/Kg exposed orally to male rats. DNA synthesis was measured in various organs and tissues by incorporation of labeled thymidine. At 125mg/Kg APAP, DNA synthesis in the rat testis was almost halved (8.5 versus 16.1 DPM/mg, 53%) one hour post treatment, and at 500mg/Kg the incorporation was almost 1/10 of control (1.7 versus 16.1 DPM/mg, 11%). The second study analyzed additional dosages and tissues, and all tested tissues showed reduced DNA synthesis following APAP exposure of ≥125mg/Kg (Table 11). This study indicates that DNA synthesis in the testis was less than half of controls at 125mg/Kg. (24.3 versus 10.9 DPM/mg). The 500mg/Kg dosage (HED 80mg/Kg, 4.8g) was reported to result in a plasma concentration of ~600μM, and a urine concentration of >9mM, 1 hour post treatment.

		Thymidine incorporation		DPM/ mg tissue	
Tissue	Control ($n = 9$)	60 mg/kg Para ($n = 3$)	125 mg/kg Para ($n = 4$)	500 mg/kg Para ($n = 4$)	1 g/kg Para ($n = 3$)
Stomach squamous	32.5 ± 16.0	19.4 ± 9.4	–	8.7 ± 1.6*	8.0 ± 0.8*
Stomach glandular	48.2 ± 12.3	47.6 ± 9.0	17.9 ± 4.1*	28.7 ± 14.8	26.2 ± 4.5*
Duodenum	129.0 ± 42.4	138.2 ± 29.3	62.9 ± 59.3*	58.1 ± 8.7*	35.2 ± 8.7*
Jejunum	223.7 ± 41.9	190.5 ± 43.7	116.1 ± 66.7*	62.0 ± 23.5*	45.2 ± 16.3*
Liver	40.4 ± 10.2	35.3 ± 13.2	27.3 ± 5.2*	44.5 ± 7.8	39.5 ± 2.2
Kidney	36.0 ± 8.3	33.3 ± 5.1	14.5 ± 3.2*	23.7 ± 4.9*	20.9 ± 1.4*
Testis	23.6 ± 7.6	24.3 ± 9.2	10.9 ± 4.9*	8.1 ± 1.8*	

Table 11. Inhibition of DNA Synthesis by Acetaminophen (Para) in Male Rats. Mean +/- SD for n separate animals. *Denotes P<O.05 as compared to the control values.[381]

Ratnasooriya (2000)

This study examined the effect of acetaminophen on the ability of male rats to reproduce.[382] The rats were given APAP (0, 500, 1000mg/Kg) orally for 30 days, and sexual behavior and fertility were tested at 2 hours after first treatment, at 30 days of treatment, and 30 days post treatment. The results showed that the rats given APAP had decreased mating and reproductive fitness (fertility index and implantation index), and this was associated with decreased sperm counts. The effect was reversible and not due to overt morbidity. The authors propose that this study supports that taking high doses of APAP for extended periods of time may reduce the ability of men to father children.

Neto (2004)

Neto et al. (2004) gave APAP (150, 500 or 1,500 mg/kg, once a day by gavage) throughout gestation (GD1-20) to pregnant Wistar rats.[383] The rats at 150mg/Kg had no reported hepatic or renal pathology,

[380] Lister and McLean. Differential inhibition of DNA synthesis by paracetamol in the rat testis and GI tract. Biochem Soc Trans. 1995 May;23(2):265S. doi: 10.1042/bst023265s. PMID: 7672290.
[381] Lister and McLean. Inhibition of DNA synthesis by paracetamol in different tissues of the rat in vivo. Toxicology. 1997 Jan 15;116(1-3):49-57. doi: 10.1016/s0300-483x(96)03521-4. PMID: 9020506.
[382] Ratnasooriya and Jayakody. Long-term administration of large doses of paracetamol impairs the reproductive competence of male rats. Asian J Androl. 2000 Dec;2(4):247-55. PMID: 11202412.
[383] Neto et al. Long-term acetaminophen (paracetamol) treatment causes liver and kidney ultra-structural changes during rat pregnancy. Clin Exp Obstet Gynecol. 2004;31(3):221-4. PMID: 15491069.

but the rats given 500 or 1500mg/Kg had liver and kidney pathologies. The authors conclude that both maternal and fetal tissues can be adversely affected by APAP.

Oyedeji (2013)

This study examined the effects of oral administration of paracetamol on hematological parameters (30 days) and reproductive parameters (21 days) in female albino rats.[384] APAP was administered by gavage at 7.5mg/Kg for 30 or 21 days based on the study endpoints. There were no significant hematological changes in the RBC, PCV, Hb, TWBC, platelet, neutrophil, lymphocyte, eosinophil, monocyte and MCH values. The duration of all phases of estrous cycle was also unaffected by the treatment of rats with APAP. The findings show that there is no effect on the blood chemistry or fertility of female albino rats at the 7.5mg/Kg/day dosage. This dosage produces a HED of 1.2mg/KG, which would be about 75mg in a 60Kg adult—only a fraction of a single recommended dose of 1000mg.

Axelstad (2014) and Kristensen (2011)

Axelstad et al (2014) reported the impact of 13 endocrine-disrupting contaminants, including pesticides, plastic and cosmetic ingredients, and APAP on Wistar rats.[385] The study examined endpoints or adverse reproductive effects, and these included decreased anogenital distance (AGD), increased nipple retention (NR), and weights of the levator ani/bulbocavernosus muscle (LABC). To avoid problems with parturition, APAP treatment (350mg/kg per day) was from GD13-19 and then reinitiated on PD14-22. The authors report that APAP exposure resulted in a significant increase in NR and reduced the weight of LABC. They also reported a non-significant reduction in AGD by 1.6%. Regarding this latter impact, the authors referenced their earlier study that indicated APAP resulted in an average reduction of 4–7% reduced AGDI in fetal rat offspring on GD 21,[386] and proposed,

> This disagreement could, however, be explained in terms of a shortened exposure duration of 7 days in this study (GD 13–19), compared with 15 days in the previous one (GD 7–21) and a different age of examination.

The dosage used in these studies (350mg/Kg) produces a HED of 56.5mg/Kg, or 3.4g in a 60Kg adult, which is 3.4 times a single 1000mg human therapeutic dose.

Johansson (2016)

This study examined the impact on female reproductive organs of rats exposed *in utero* and postnatally to APAP (at 360 mg/Kg).[387] In pre-pubertal rats, the authors report that there was a reduction in primordial follicle numbers due to APAP exposures. In mature dams (1yr), they also reported decreased ovary

[384] K.O, Oyedeji. "Evaluation of Haematological and Reproductive Effect of Paracetamol (Acetaminophen) In Female Abino Rats." IOSR Journal of Dental and Medical Sciences 3.5 (2013): 72–75. Web.
[385] Axelstad M, et al. Mixtures of endocrine-disrupting contaminants induce adverse developmental effects in preweaning rats. Reproduction. 2014 Mar 2;147(4):489-501. doi: 10.1530/REP-13-0447. PMID: 24298046.
[386] Kristensen et al. Intrauterine exposure to mild analgesics is a risk factor for development of male reproductive disorders in human and rat. Hum Reprod. 2011 Jan;26(1):235-44. doi: 10.1093/humrep/deq323. Epub 2010 Nov 8. PMID: 21059752.
[387] Johansson et al. Perinatal exposure to mixtures of endocrine disrupting chemicals reduces female rat follicle reserves and accelerates reproductive aging. Reprod Toxicol. 2016 Jun;61:186-94. doi: 10.1016/j.reprotox.2016.03.045. Epub 2016 Apr 2. PMID: 27049580.

weights and suggest that their findings resemble premature ovarian insufficiency in humans and raise concerns regarding female reproductive function due to perinatal APAP exposures.

Hurtado-Gonzalez et al. (2018)

This study used multiple *in vitro* and *in vivo* models to examine the impact of APAP of gonadal cells and tissues.[388] They used first-trimester human fetal testes and ovaries for *in vitro* testing. They also incorporated second-trimester fetal germ cells in rats to produce a xenograft model. The number of gonocytes was reduced relative to controls in first-trimester human fetal testes and ovaries exposed to APAP. After treatment of xenograft host mice for one day or seven days, APAP reduced gonocyte number by up to 30%. The expression of GC pluripotency genes and genes that regulate DNA/histone methylation were altered by APAP. The authors report consistently increased expression of the epigenetic regulator TET1 in APAP exposed human NTera2 cells, rat fetal testis/ovary cultures, and in fetal testes and ovaries. They conclude that these results raise concerns about the use of APAP during human pregnancies.

Yilmaz (2020)

This study showed that *in utero* exposure of rats to APAP (50, 125, 250, 500mg/Kg) throughout gestation (GD1-20) produced various changes in the fetal liver.[389] Specifically, vascular endothelial growth factor A (VEGF-A) and FETU-A (FETUIN-A) had increasing responses from 0-500mg/Kg and Sclerostin (SOST) significantly increased at 125 and 250, but fell at 500mg/Kg.

Pereira (2020)

A study design similar to Klein 2020 was used by Pereira et al. to examine the impact of *in utero* exposure (GD6-21) versus perinatal exposure (GD6-PND21) to APAP (350 mg/kg/day) on the sexual behavior of adult male rats.[390] There was no overt toxicity with this treatment and no statistical difference between the reproductive organs, kidneys, adrenals, liver, or pituitary weight in all ages. There were also no statistical differences observed in the sperm count, morphology, or spermatic motility. The authors did report significantly increased testosterone levels, volumes and total lengths of the seminiferous tubules in both gestational and perinatal APAP exposed groups at PND 120. The authors conclude that maternal exposure to APAP has an impact on the reproductive system and sexual behavior of male adult offspring and may impair sexual hypothalamic differentiation during brain development.

Aleixo (2020)

[388] Hurtado-Gonzalez et al. Effects of Exposure to Acetaminophen and Ibuprofen on Fetal Germ Cell Development in Both Sexes in Rodent and Human Using Multiple Experimental Systems. Environ Health Perspect. 2018 Apr 16;126(4):047006. doi: 10.1289/EHP2307. PMID: 29665328; PMCID: PMC6071829.

[389] Yilmaz S, Göçmen YA, Tokpınar A. et al. Effects of Paracetamol on Vascular Endothelial Growth Factor, Sclerostin and FETUIN-A in the Liver of Rat Fetuses. Acta Med. Alanya 2020;4(2):150-155. doi:10.30565/medalanya.688286

[390] Pereira et al. Can maternal exposure to paracetamol impair reproductive parameters of male rat offspring? Reprod Toxicol. 2020 Apr;93:68-74. doi: 10.1016/j.reprotox.2019.12.007. Epub 2020 Jan 9. PMID: 31926975.

Aleixo et al. performed a study on pregnant Wistar rats gavaged daily with APAP (350mg/Kg) or water from GD6 until delivery. Maternal APAP treatment resulted in increased grooming behavior. Impacts on offspring included impaired sexual behavior and decreased egg follicle reserve in female offspring.[391]

5. Other Acetaminophen Rat Studies

In this section, I briefly describe several rat studies involving acetaminophen that do not fall into the other categories above.

Lubawy 1977

This study tested the effects of aspirin and APAP (0, 125, 250mg/Kg) on pregnant rats.[392] The medications were given to the rats by gavage for 12 days during pregnancy (GD8-19). The animals were terminated on GD20, and the researchers determined fetal and placenta weights. The was no reported gross pathology. Litters from the rats given acetaminophen at 125mg/Kg had an increased incidence of resorptions (16% vs 0% in controls) and the pups were also smaller in length at this dosage. The higher dosage of APAP showed non-significant increases in pregnancy weights for dams and fetuses, and resorptions were not increased. The authors suggest caution for interpretation of the results in humans, and report that APAP produced adverse effects relative to controls, but these were less severe than those observed with aspirin.

Lin 1983

This study examined the pharmacokinetics of APAP in pregnant versus non-pregnant rats at two dosages, 15 and 300mg/Kg given IV.[393] The researchers treated the pregnant rats on GD20 and collected blood and urine samples to examine APAP metabolites. The authors found that pregnant rats processed the 300mg/Kg dose of APAP differently than non-pregnant rats, with a longer half-life and slower clearance (Figure 31). Pregnant rats also excreted more unmetabolized APAP, produced less APAP-sulfate and the same fraction of APAP-glucuronide as did nonpregnant animals. However, pregnancy was not reported to affect the base-line serum inorganic sulfate concentration. The investigators also reported that both non-pregnant and pregnant rats became inorganic sulfate-depleted at the 300 mg/kg dosage. This dosage in rats produces a HED of ~48mg/Kg, or ~3g for a 60Kg reference adult human. Overall, this study indicates that pregnancy can affect how the body metabolizes APAP.

[391] Aleixo et al. Effect of paracetamol treatment on maternal care and reproductive outcomes in female rat offspring. Reprod Fertil Dev. 2020 Dec;32(18):1311-1325. doi: 10.1071/RD20007. PMID: 33308393.
[392] Lubawy and Garrett. Effects of aspirin and acetaminophen on fetal and placental growth in rats. J Pharm Sci. 1977 Jan;66(1):111-3. doi: 10.1002/jps.2600660129. PMID: 833724.
[393] Lin and Levy. Effect of pregnancy on the pharmacokinetics of acetaminophen in rats. J Pharmacol Exp Ther. 1983 Jun;225(3):653-9. PMID: 6602874.

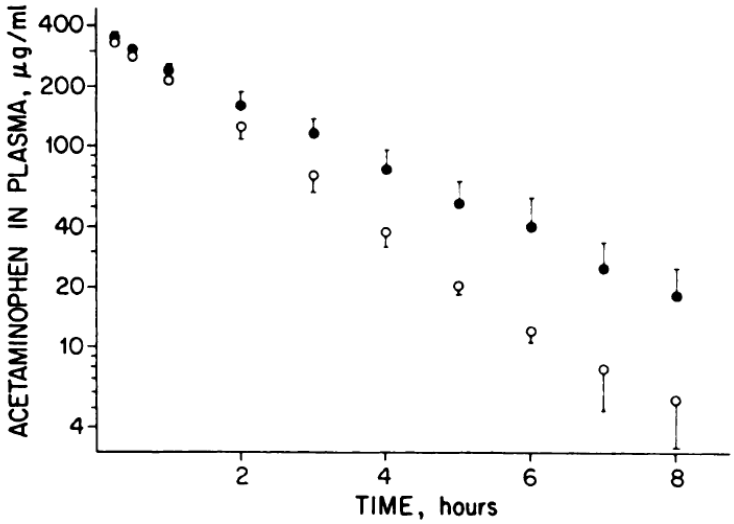


Figure 31. Acetaminophen Metabolism in Pregnant Rats. Acetaminophen concentrations in plasma of 20 days pregnant rats (●) and nonpregnant female rats (○) after i.v. injection of 300 mg/kg. Vertical bars represent 1 S.D. n=7/group.[393]

Koehn (2021)

Adenosine triphosphate binding cassette (ABC) transporters are involved in drug pharmacokinetics and can support homeostatic cellular functioning by removing certain molecules and metabolites from a cell. They can also limit the access of drugs to active sites located within a cell or restrict the transport of drugs across cellular interfaces. By maintaining desirable concentrations of substrate molecules in different areas of the body, these transporters perform endogenous functions and provide protection from a range of xenobiotics.[394] The Koehn et al. study examined whether *in utero* APAP exposure had effects on ABC transporters.[395] The investigators administered APAP (IP) twice daily to Sprague-Dawley rats from embryonic day E15 to E19 (chronic) or as a single dose at E19 (acute) at 3.75mg/Kg or 15mg/Kg. RNAseq analysis identified transcripts for ABC transporters and enzymes in rat E19, P5, and adult brain and choroid plexus and E19 placenta. The results showed that the transcription of many ABC transporters was higher in the adult brain of rats exposed to APAP *in utero*. Chronic exposure also resulted in increased APAP in the brain (E19, high dose) and plasma of dams and fetuses (E19, high dose). The authors conclude that the transfer of APAP from blood to brain differs by developmental timing, dose, and duration.

E. Hamster and Non-Rodent Animal Studies

The study by Rutkowski and Ferm (1982) evaluated the teratogenic effects of the isomeric forms of aminophenol (para-AP/p-AP, meta-AP/m-AP, and ortho-AP/o-AP) in Syrian golden hamsters.[396] As indicated previously, p-AP is a metabolite of APAP, that can be metabolized into AM404, a modifier of cannabinoid signaling (see **Cannabinoid Interactions**, above). The hamsters were given a dose of 100 to

[394] Higgins. ABC Transporters: Physiology, Structure and Mechanism – an Overview. Research in microbiology 152.3 (2001): 205–210. Web.

[395] Koehn et al. Efflux transporters in rat placenta and developing brain: transcriptomic and functional response to paracetamol. Sci Rep. 2021 Oct 6;11(1):19878. doi: 10.1038/s41598-021-99139-6. PMID: 34615937; PMCID: PMC8494792.

[396] Rutkowski and Ferm. Comparison of the teratogenic effects of the isomeric forms of aminophenol in the Syrian golden hamster. Toxicol Appl Pharmacol. 1982 Apr;63(2):264-9. doi: 10.1016/0041-008x(82)90048-5. PMID: 7089975.

200 mg/kg of p-AP, m-AP, or o-AP on Day 8 of gestation and were terminated on Day 13. The study reported that p-AP and o-AP produced a significant dose-responsive teratogenic response, with an increase in the frequency of litters with one or more malformed fetuses and an increase in the number of fetuses with one or more malformations as the dose increased. Common malformations observed included exencephaly, encephalocele, eye defects, rib fusion, tail defects, spina bifida, limb defects, and umbilical hernia. The data supports the conclusion that p-AP and o-AP are teratogenic in Syrian golden hamsters without compromising maternal health. As indicated in the discussion of the study,

> Maternal health was not compromised following treatment with increasing doses of p-AP, o-AP, or m-AP. Normal levels of activity and appetite were maintained by the dams. There was no evidence of maternal disease, characterized by listlessness, hair loss, diarrhea, or overt weight loss, during the course of experimentation. The teratogenicity of p-AP may be related to the formation of a reactive quinone metabolite which can readily bind macromolecules.

This study lacks replication, as there were no additional developmental toxicity studies of APAP in Syrian golden hamsters.

F. Labeling of APAP Safety During Pregnancy

As a teratologist, I am familiar with the FDA pregnancy categories that were required for prescription drugs from 1979 until 2015[397].The pregnancy categories incorporate work done in the field of teratology to identify the teratogenic potential of pharmaceuticals taken in pregnancy. Over the course of my career at medical institutions within the Texas Medical Center and at Dell Pediatric Research Institute I have been routinely consulted by clinicians about the safety of various medications in pregnancy, including the various pregnancy categories. I have conducted drug safety studies that were submitted to the FDA for pregnancy category labeling support. The pregnancy categories were used by healthcare providers to quickly assess the safety of drugs in pregnancy. There were five categories – A, B, C, D, and X – that summarized a medication's risk to the fetus as described in the table below:

[397] Pernia S, DeMaagd G. The New Pregnancy and Lactation Labeling Rule. P T. 2016 Nov;41(11):713-715. PMID: 27904304; PMCID: PMC5083079; Gruber M. F. The FDA pregnancy lactation and labeling rule – Implications for maternal immunization.Vaccine, 33 (2015), pp. 6499-6500, 10.1016/j.vaccine.2015.05.107.

A	Adequate and well-controlled (AWC) studies in pregnant women have failed to demonstrate a risk to the fetus in the first trimester of pregnancy (and there is no evidence of a risk in later trimesters).
B	Animal reproduction studies have failed to demonstrate a risk to the fetus and there are no AWC studies in pregnant women, OR animal studies demonstrate a risk and AWC studies in pregnant women have not during the first trimester (and there is no evidence of risk in later trimesters).
C	Animal reproduction studies have shown an adverse effect on the fetus, there are no AWC studies in humans, AND the benefits from the use of the drug in pregnant women may be acceptable despite its potential risks. **OR** animal studies have not been conducted and there are no AWC studies in humans.
D	There is positive evidence of human fetal risk based on adverse reaction data from investigational or marketing experience or studies in humans, BUT the potential benefits from the use of the drug in pregnant women may be acceptable despite its potential risks (for example, if the drug is needed in a life-threatening situation or serious disease for which safer drugs cannot be used or are ineffective).
X	Studies in animals or humans have demonstrated fetal abnormalities OR there is positive evidence of fetal risk based on adverse reaction reports from investigational or marketing experience, or both, AND the risk of the use of the drug in a pregnant woman clearly outweighs any possible benefit (for example, safer drugs or other forms of therapy are available).

Table 11. FDA Pregnancy Categories[398]

Rarely would a drug be assigned Category A since it is almost always unethical to perform controlled studies in pregnant women. As a result, Category B was generally presumed to be safe in pregnancy. Conversely, Category C was generally accepted as not safe in pregnancy requiring healthcare providers to consider the seriousness of the condition to be treated and the risk of not treating the condition relative to the risk to the fetus.

I reviewed the label for the prescription drug Ofirmev. In 2009, Cadence Pharmaceuticals, Inc. submitted a New Drug Application for an intravenous APAP drug, Ofirmev, prompting the FDA to evaluate APAP's teratogenic risks and designate its pregnancy category *for the first time*.[399] I reviewed the comments of the Ofirmev Maternal Health Team (MHT) and Pharmacology reviewers in connection with the consideration of the Ofirmev application and labeling. The Ofirmev MHT Review states, "Acetaminophen was approved by the FDA in 1951, prior to the 1979 regulations that established pregnancy categories, **and there are no approved acetaminophen containing products labeled with a pregnancy category based on acetaminophen content** and any acetaminophen-associated risk." (emphasis added)[400] One of the Ofirmev Pharmacology reviewers commented, "To my knowledge, a drug product label for acetaminophen as a stand alone prescription drug product has not previously been approved by the FDA."[401]

[398] https://www.fda.gov/media/133262/download

[399] Prior to 2010, FDA approved and assigned pregnancy categories to combination APAP drugs, including Vicodin, Ultracet and Percocet. However, prior to 2010, "there [were] no approved acetaminophen-containing products labeled with a pregnancy category *based on acetaminophen content and acetaminophen-associated risk*." Ofirmev Other Review(s) at 8

[400] Ofirmev Maternal Health Team (MHT) Review at 3

[401] Ofirmev Pharmacology Review(s) at 5

The label of Ofirmev includes data from testing in mice. Briefly, the Ofirmev label documents reduced fecundity and abnormal sperm resulting from oral APAP exposure in mice at clinically relevant dosages, based on maximum human daily dosages (MHDD):

In a continuous breeding study, pregnant mice received 0.25, 0.5, or 1.0% acetaminophen via the diet (357, 715, or 1430 mg/kg/day). These doses are approximately 0.43, 0.87, and 1.7 times the MHDD, respectively, based on a body surface area comparison. A dose-related reduction in body weights of fourth and fifth litter offspring of the treated mating pair occurred during lactation and post-weaning at all doses. Animals in the high dose group had a reduced number of litters per mating pair, male offspring with an increased percentage of abnormal sperm, and reduced birth weights in the next generation pups.
This label documents "reduced number of litters per mating pair" which indicates a reduced capacity to produce offspring at a therapeutically relevant exposure (1.7X MHDD) in mice. In my opinion, a "reduced number of litters per mating pair" is consistent with a reduced capacity to produce offspring and "increased percentage of abnormal sperm" are evidence of reproductive toxicity and would not permit a pregnancy category B designation.

The Ofirmev label also documents fetotoxicity resulting from oral APAP exposure in rats at clinically relevant dosages:

> While animal reproduction studies have not been conducted with intravenous acetaminophen, studies in pregnant rats that received **oral** acetaminophen during organogenesis at doses up to 0.85 times the maximum human daily dose (MHDD = 4 grams/day, based on a body surface area comparison) showed evidence of fetotoxicity (reduced fetal weight and length) and a dose-related increase in bone variations (reduced ossification and rudimentary rib changes). Offspring had no evidence of external, visceral, or skeletal malformations. When pregnant rats received oral acetaminophen throughout gestation at doses of 1.2-times the MHDD (based on a body surface area comparison), areas of necrosis occurred in both the liver and kidney of pregnant rats and fetuses. These effects did not occur in animals that received oral acetaminophen at doses 0.3-times the MHDD, based on a body surface area comparison.[402]

This label also documents "evidence of fetotoxicity" at a therapeutically relevant exposure (0.85X MHDD) in rats. Ofirmev was approved by the FDA on November 2, 2010. OTC APAP, including Tylenol, which contains the same active ingredient acetaminophen (APAP) was never shown to be safe in preclinical safety or NTP animal studies, and preclinical studies never supported pregnancy category B labeling. Pregnancy category B indicates that animal reproduction studies have not demonstrated a risk to the fetus, but there are no well-controlled studies in pregnant women. I opine that "evidence of fetotoxicity" is not the same as "animal reproduction studies have not demonstrated a risk to the fetus." The Ofirmev label states: "Pregnancy: Category C" and "Use only if clearly needed."[403]

[402] Ofirmev Label (rev. 11/2010).
[403] Ofirmev Label (rev. 11/2010).

This designation is further described on the Ofirmev label, which explains there are "no studies of intravenous acetaminophen in pregnant women" and "[a]nimal reproduction studies [had] not been conducted with IV acetaminophen".[404] In fact, the Ofirmev label's Pregnancy section only references epidemiological and preclinical studies of *orally* administered APAP. Therefore, to the extent the FDA evaluated the teratogenic risks of stand-alone, oral APAP, it determined those risks are appropriately described by pregnancy category C.

I also reviewed the label for Ultracet as well as the publicly available labeling history on the FDA's website, a combination prescription oral drug containing acetaminophen and tramadol hydrochloride.[405] Ultracet was first approved by the FDA in 2001 and was designated as pregnancy category C.[406] Ultracet is manufactured by Janssen Pharmaceutical Companies, a Johnson & Johnson company. Although, unlike Ofirmev, Ultracet is a combination drug, the Ultracet label was modified at some point between October 18, 2013 and December 16, 2016 to specifically include animal data specific to stand-alone acetaminophen.[407] An excerpt from the 2016 Ultracet Label is shown below:

> Reproductive and developmental studies in rats and mice from the published literature identified adverse events at clinically relevant doses with acetaminophen. Treatment of pregnant rats with doses of acetaminophen approximately 1.3 times the maximum human daily dose (MRHD) showed evidence of fetotoxicity and increases in bone variations in the fetuses. In another study, necrosis was observed in the liver and kidney of both pregnant rats and fetuses at doses approximately 1.9 times the MHDD. In mice treated with acetaminophen at doses within the clinical dosing range, cumulative adverse effects on reproduction were seen in a continuous breeding study. A reduction in number of litters of the parental mating pair was observed as well as retarded growth and abnormal sperm in their offspring and reduced birth weight in the next generation *[see Data]*. Based on animal data, advise pregnant women of the potential risk to a fetus.

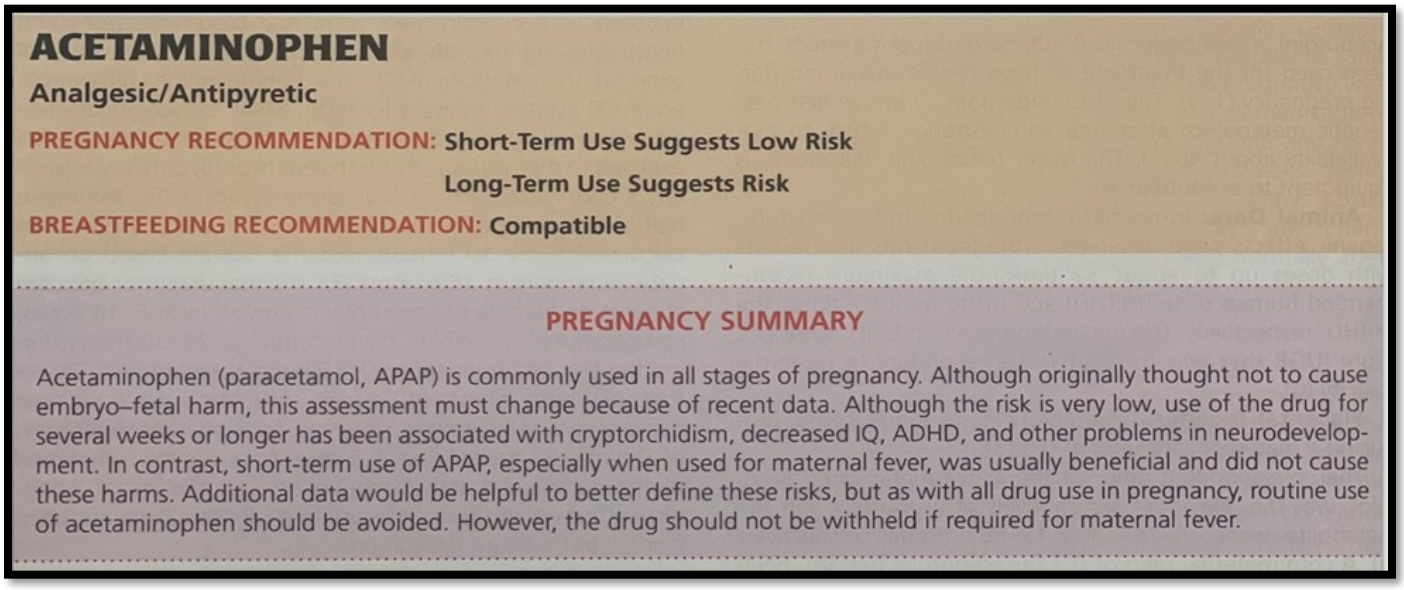
The 2023 Ultracet Label contains the same APAP-specific language as the 2016 version.[408] This label documents "reduction in number of litters," "retarded growth and abnormal sperm" in offspring and "reduced birth weight in the next generation." The endpoints clearly support reproductive and developmental toxicity and are consistent with "manifestations of deviant development", as defined in Wilson's Principles. In short, since 2016, a Johnson & Johnson prescription pharmaceutical drug containing APAP has contained references to animal studies showing adverse effects on the fetus at clinically relevant doses of acetaminophen and a statement that pregnant women should be advised of the potential risks to a fetus based upon this animal data.

I reviewed deposition transcripts and exhibits of JJCI witnesses. Even though the FDA replaced the traditional pregnancy categories in 2015, [redacted]

[404] *Id*. at § 8.1
[405] Ultracet Label (rev. 2/2023).
[406] https://www.accessdata.fda.gov/scripts/cder/daf/index.cfm?event=BasicSearch.process
[407] Ultracet Label (rev. 12/2016);
[408] Ultracet Label (rev. 2/2023).

[redacted]

I reviewed the Acetaminophen section of Briggs 2022, which states, "Acetaminophen (paracetamol, APAP) is commonly used in all stages of pregnancy. **Although originally thought not to cause embryo-fetal harm, this assessment must change because of recent data**." (emphasis added). The pertinent portions of Briggs 2022 are shown below:



ACETAMINOPHEN

Analgesic/Antipyretic

PREGNANCY RECOMMENDATION: **Short-Term Use Suggests Low Risk**
Long-Term Use Suggests Risk

BREASTFEEDING RECOMMENDATION: **Compatible**

PREGNANCY SUMMARY

Acetaminophen (paracetamol, APAP) is commonly used in all stages of pregnancy. Although originally thought not to cause embryo–fetal harm, this assessment must change because of recent data. Although the risk is very low, use of the drug for several weeks or longer has been associated with cryptorchidism, decreased IQ, ADHD, and other problems in neurodevelopment. In contrast, short-term use of APAP, especially when used for maternal fever, was usually beneficial and did not cause these harms. Additional data would be helpful to better define these risks, but as with all drug use in pregnancy, routine use of acetaminophen should be avoided. However, the drug should not be withheld if required for maternal fever.

[redacted] Candeias-Hernandez et al. published a systematic review with citation tracking that examined the history of experiments on the safety of acetaminophen.[413] The study authors showed that there were abundant studies demonstrating that, at therapeutic doses, APAP did not pose a significant risk of pediatric liver toxicity, but the authors also reported that there were no published studies demonstrating the safety of APAP during neurodevelopment.

[redacted]

[410] Briggs, G., et al, (1990) Drugs in Pregnancy and Lactation: A Reference Guide to Fetal and Neonatal Risk (3rd edition).

[411] Briggs, G., et al, (2022) Drugs in Pregnancy and Lactation: A Reference Guide to Fetal and Neonatal Risk (12th edition).

[redacted]

[413] Cendejas-Hernandez et al. Paracetamol (acetaminophen) use in infants and children was never shown to be safe for neurodevelopment: a systematic review with citation tracking. Eur J Pediatr. 2022 May;181(5):1835-1857. doi: 10.1007/s00431-022-04407-w. Epub 2022 Feb 17. PMID: 35175416; PMCID: PMC9056471

Of the 52 studies that described experiments designed to test the safety of APAP in infants or children, most examined only liver toxicity. Of the five studies that monitored outcomes 28 or more days after APAP exposure, none of them monitored neuropsychiatric function (see Fig. 2 in Candeias-Hernandez et al.) In the absence of adequate and well-controlled studies in pregnant women coupled with the animal studies showing adverse effects on the fetus as detailed in the Ofirmev and Ultracet labels, APAP would not meet the criteria for pregnancy category B.

[redacted]

In the field of teratology, which seeks to minimize the risk of birth defects through education, research, and public health initiatives, the FDA pregnancy categories were a tool by which pregnant women and their healthcare providers could readily evaluate the potential teratogenic risks associated with a drug in deciding whether the potential risks outweighed the benefits. The lack of consistency in the teratogenic risk information presented for APAP products undermines the goals of teratology and prohibits pregnant women from accessing the information they need to make informed decisions.

[redacted]

G. AOP and WoE Based Conclusion from Animal Studies

Several conclusions can be drawn from these animal studies. These conclusions are presented based on applying the weight of evidence (WoE) to the Oxidative Stress Adverse Outcome Pathway (AOP) that identifies APAP as a stressor.

The NTP (National Toxicology Program) has published guidelines for rating the strength of the evidence for drawing conclusions from a study.[415] The guidelines define five levels of evidence:

"Levels of Evidence for Evaluating Developmental System Toxicity

- **Clear evidence of developmental toxicity** is demonstrated by data that indicate a dose-related effect on one or more of its four elements (embryo-fetal death, structural malformations, growth retardation or functional deficits) that is not secondary to overt maternal toxicity.
- **Some evidence of developmental toxicity** is demonstrated by dose-related effects on one or more of its four elements (embryo-fetal death, structural malformations, growth retardation or functional deficits), but there are greater uncertainties or weaker relationships with regard to dose, severity, magnitude, incidence, persistence, and/or decreased concordance among affected end points.
- **Equivocal evidence of developmental toxicity** is demonstrated by marginal or discordant effects on developmental parameters that may or may not be related to the test article.
- **No evidence of developmental toxicity** is demonstrated by data from a study with appropriate experimental design and conduct that are interpreted as showing no biologically relevant effects on developmental parameters that are related to the test article.
- **Inadequate study of developmental toxicity** is demonstrated by a study that, because of major design or performance flaws, cannot be used to determine the occurrence of developmental toxicity."

Conclusion 1: Perinatal APAP exposure via NAPQI is reported to decrease GSH and increase adduct formation.

Exposing mice and rats during development to acetaminophen at human equivalent doses causes significantly reduced glutathione levels and significantly increased oxidative stress, including in the brain. Multiple studies in both mice and rats demonstrate these effects. There is "clear evidence" supporting dose-responsive, physical-chemical interactions between NAPQI and GSH and protein-thiols. These studies and supporting data are peer-reviewed, replicated, and include high quality regulatory studies. This AOP interaction is supported based on the WoE.

Conclusion 2: Perinatal APAP exposure via NAPQI is reported to cause oxidative stress.

Exposing mice and rats during development to acetaminophen at human equivalent doses causes significantly increased oxidative stress. Oxidative stress is measured and supported by standardized assays, including TBARS. There is "clear evidence" supporting dose-responsive production of oxidative stress by NAPQI. These studies and supporting data are peer-reviewed, replicated, and include high quality regulatory studies. This AOP interaction is supported based on the WoE.

Conclusion 3: Perinatal APAP exposure via NAPQI is reported to result in neurotoxicity.

[415] NTP- Explanation of Levels of Evidence for Developmental Toxicity. http://ntp.niehs.nih.gov/go/10003. Paul M. Foster.

Exposing mice and rats during development to acetaminophen at human equivalent doses during neural development causes significant neurodevelopmental impacts to mice and rats. A total of 22 studies (8 mice and 14 rats) investigated whether early-life exposure to acetaminophen caused biological changes in the brain. Animal studies show "clear evidence" of developmental toxicity, consistent with regulatory guidelines. The evidence of developmental toxicity is strengthened because multiple studies show increases in severity and/or prevalence as a function of dose (dose responsive toxicity was observed in multiple studies), effects seen in litter-based analyses provide stronger evidence (multiple litters impacted across multiple studies), and concordant effects (oxidative damage causing DNA oxidation, reproductive toxicity, and neurodevelopment toxicity). Finally, consistent with "clear evidence" of developmental toxicity, these end points are reported as statistically and biologically significant across multiple litters tested and multiple studies.[416]

The following animal studies provide "clear evidence" of developmental toxicity, with oxidative stress and oxidative DNA damage, consistent with developmental neurotoxicity due to perinatal APAP exposure.

Mice.

Animal Study	Year	Evidence
Ruepp	2002	decreased glutathione, loss of ATP production, and enlargement and fusion of mitochondria
De Silva	2012	systemic depletion of glutathione and resulting brain damage
Markovic	2013	decreased glutathione and DNA damage consistent with oxidative stress
Viberg	2014	changes in the levels of brain derived neurotropic factor
Hay-Schmidt	2017	decrease in the number of neurons in a portion of the hypothalamus
Philippot	2018	reduction in cerebral cortical fatty acid amide hydroxylase (FAAH), and reduction of transcript levels of hippocampal synaptophysin (Syp) and tropomyosin receptor kinase B
Philippot	2022	acute oxidative stress in the hippocampus
Baker	2023	altered prefrontal cortex gene expression related to various molecular pathways

Rats.

Animal Study	Year	Evidence
Harris	1989	abnormal closure of the anterior neuropore
Dean	2012	increased spinolin and dendritic growth
Blecharz-Klin	2013	levels of monoamines and metabolites
Blecharz-Klin	2014	decreased amino acids in the striatum (glutamine, glutamic acid, taurine, alanine, aspartic acid) and hypothalamus (glycine); increased levels of γ-aminobutyric acid (GABA) in the prefrontal cortex
Blecharz-Klin	2015a	increased aspartic and glutamic acid in the spinal cord

[416] NTP, Explanation of Levels of Evidence for Developmental Toxicity (http://ntp.niehs.nih.gov/go/10003)

Blecharz-Klin	2015b	altered dopaminergic system in the medulla oblongata
Lichtensteiger	2015	altered gene expression in the hypothalamus
Blecharz-Klin	2017	amino acid levels in the hippocampus
Blecharz-Klin	2018	decreased BDNF in the striatum
Blecharz-Klin	2019	altered levels of monoamines, metabolites, amino acids, dopamine, and noradrenaline in the hypothalamus
Saeedan	2018	increase increased oxidative markers (TBARS) and inflammatory markers
Klein	2020	reduced glutathione and brain derived neurotrophic factor
Koehn	2020	increased cytokines and immune-response genes, and reduced acute phase plasma proteins
Rigobello	2021	decreased glutathione

Perinatal APAP exposure is reported to impair learning or social behavior.

Exposing mice and rats during development to APAP at human equivalent doses during neural development causes significantly altered learning, locomotor, and social behavior consistent with ASD and ADHD. A total of 17 studies (10 in mice and 7 in rats) investigated whether early-life exposure to acetaminophen caused behavioral changes consistent with ASD and ADHD. Of these, 15 of the 17 showed that APAP caused impaired learning and altered behaviors.[417] Accordingly, these studies provide both "clear evidence" and "some evidence" of developmental toxicity. The other 2 studies used low dosages (Blecharz-Klin 2017) or multifactorial non-DART experiments (Zhao 2017) that showed "no evidence" in the WoE for impaired learning or social behavior. The "no evidence" is proposed to be a result of the sub-therapeutic dose in the former study. The studies by Zhao include multiple variables that make interpretation a circular paradox, often termed begging the question, this includes LPS causing inflammation and fevers, APAP causing toxicity, APAP reducing inflammation or fever reduction, reported LPS mitigation of APAP toxicity, and reciprocated APAP mediation of LPS toxicity. As both agents potentiate toxicity but mitigate the toxicity of each other, "no evidence" is one expected outcome. Consistent with neurobehavioral study guidelines, these animal studies show "clear evidence" that perinatal APAP exposure results in learning deficits and impaired social behavior.

Mice

Animal Study	Year	Evidence
Ishida	2017	clear evidence of learning deficits
Gould	2012	clear evidence of repetitive behavior, social interactions
Viberg	2014	clear evidence of ambulation and learning deficits

[417] In addition, Diaz-Estrada 2021 concluded that acetaminophen exposure during neurodevelopment caused males to have increased homosexual partner preference, and Hay-Schmidt 2017 found that acetaminophen exposure resulted in altered sexual behavior.

Saad	2016	clear evidence of locomotor activity, ambulation, fine repetitive movements
Zhao	2017	Inadequate study of developmental toxicity. The 2017 study was conducted in adult mice treated with LPS or APAP, and APAP 100mg/Kg did not differ from control in behavioral testing in the absence of LPS.
Philippot	2017	clear evidence of deficit in habituation capability
Philippot	2018	clear evidence of altered spontaneous behavior and habituation capability in a novel home cage
Philippot	2022	clear evidence of impaired habituation capability, reduced memory and learning ability, and decreased cognitive flexibility
Harshaw	2022	clear evidence of impaired social-emotional and repetitive behaviors
Baker	2023	clear evidence of impaired social behavior

Rats

Animal Study	Year	Evidence
Dean	2012	clear evidence of social interaction and sensory threshold in males
Blecharz-Klin	2017	equivocal evidence, alterations in cognitive functions, improved spatial memory and search strategy at sub-therapeutic doses (5mg/Kg), but no significant differences at increased dosing (15mg/Kg).
Blecharz-Klin	2018	clear evidence of a lower frequency of social interactions, greater pinning behavior, and rats from the 15mg/kg group exhibited a greater interest in objects in the novel object recognition tests.
Saeedan	2018	some evidence of impaired eye opening, locomotor activity, olfactory discrimination, and on entries in the elevated plus maze.
Klein	2020	clear evidence of impaired nest seeking behavior and decreased rostral grooming in the elevated plus maze.
Rigobello	2021	clear evidence of augmented stereotyped behavior and ambulation, reduction in exploratory behavior.
Suda	2021	clear evidence of increased asocial rearing behavior

Conclusion 5: Perinatal APAP exposure is reported to cause developmental or reproductive toxicity.

Acetaminophen exposure during reproductive and developmental toxicity testing caused both mice and rats to have alterations in reproductive and other tissues. These effects were consistent across multiple studies.

Conclusion 6: Perinatal APAP exposure shows dose, duration, and frequency dependent interactions.

The effects described above by exposing acetaminophen were dose and duration dependent. Exposure to greater doses and for longer durations increased the effects on brain tissues and behavior.

Conclusion 7: Perinatal APAP exposure is reported to cause specific and consistent toxicities.

The effects on brain tissues and behavior show a high degree of consistency, with over 90% of the studies supporting the hypothesis that APAP exposure during neurodevelopment at doses equivalent to therapeutic human doses causes significant changes in the brain and results in significant behavioral effects in later life.

5. Population (Human)

Reflective and supportive of the above-discussed animal studies, the scientific literature also supports increased risk of neurodevelopmental toxicity in humans associated with APAP exposure during pregnancy. Reported interactions between APAP and developmental toxicity endpoints include autism spectrum disorder (ASD) and attention deficit hyperactivity disorder (ADHD). The available epidemiological evidence is reviewed systematically and is organized into the following six categories:

- APAP with ASD
- APAP with ADHD
- APAP with impaired learning, cognitive, or social outcomes
- Meta-analyses of the Association of APAP with ASD or ADHD
- Other relevant reviews
- APAP and other birth outcomes

A. Studies Examining the Association of APAP with ASD

Avella-Garcia 2016

The study is a Spanish birth cohort study that investigated the association between prenatal exposure to acetaminophen and neurodevelopmental outcomes at 1 and 5 years of age.[418]

Information used to determine exposure: The exposure of interest was acetaminophen use during pregnancy, which was assessed using two structured interviews that asked about medication use during pregnancy. Ever/never use and frequency of use (never, sporadic, persistent) were measured.

Outcome definition and determination: The main neurodevelopmental outcomes were assessed using the Childhood Autism Spectrum Test (CAST), Conner's Kiddie Continuous Performance Test (K-CPT), and ADHD-DSM-IV form list.

Control group: The control group consisted of children whose mothers did not report using APAP during pregnancy.

Study size: The study included 2644 mother-child pairs recruited during pregnancy. The proportion of liveborn participants evaluated at 1 and 5 years was 88.8% and 79.9%, respectively.

Confounding factors or biases and how or if they were controlled: The study controlled for several potential confounding factors, including social determinants and co-morbidities. Regression models were adjusted for these factors.

Limitations: Reported limitations included confounding by indication, despite adjustment, genetic confounding, residual confounding, self-reporting, and potential misclassification.

[418] Avella-Garcia et al. Acetaminophen use in pregnancy and neurodevelopment: attention function and autism spectrum symptoms. Int J Epidemiol. 2016 Dec 1;45(6):1987-1996. doi: 10.1093/ije/dyw115. PMID: 27353198.

Results: The study found that prenatal acetaminophen exposure was associated with a greater number of autism spectrum symptoms in males and showed adverse effects on attention-related outcomes for both genders. In this study, 43% of children evaluated at age 1 (n = 2195) and 41% of those assessed at age 5 (n = 2001) were exposed to APAP. Exposed children showed a higher risk of presenting hyperactivity/impulsivity symptoms than non-exposed children (incidence rate ratio (IRR) = 1.41, 95% CI 1.01–1.98), a greater risk of K-CPT commission errors (IRR = 1.10, 95% CI 1.03–1.17) and lower detectability scores (β = 0.75, 95% CI 0.13–0.02), than non-exposed children after full multivariate adjustment.

In conclusion, this study found that taking acetaminophen during pregnancy may lead to more symptoms of Autism Spectrum Condition (ASC) in male children. It also showed that prenatal exposure to this medication can affect attention function in 5-year-old children, but the effects may be different for boys and girls. The study also found a connection between acetaminophen exposure and hyperactivity/impulsivity behaviors in all children.

Bittker 2018

The study design was an internet-based survey.[419]

Information used to determine exposure: Participants were parents living in the USA with at least one biological child between 3 and 12 years of age. Potential participants were informed about the survey via postings on social media, websites, and listservs and were offered an opportunity to participate in a raffle as well. Participants were also recruited through the Interactive Autism Network.

Study size: After exclusions, there remained 1,001 responses associated with children with ASD (cases) and 514 responses associated with children who do not have ASD (controls). The outcome definition was whether or not a child had ASD.

Confounding factors or biases and how or if they were controlled: The study adjusted for eight demographic variables when analyzing the association between the postnatal variables and ASD. Adjusted variables, e.g., education, as a scaled variable, race as binary 0 (White (non-Hispanic)) or 1 (other). Specific confounding factors or biases that were controlled for are not clerly indicated.

Limitations: Reported limitations include being an internet survey, population structure differences including gender distribution, mean age of child, maternal education, and, to a lesser extent, ethnicity.

Results: In this study, doses of postnatal acetaminophen, adjusted odds ratio (aOR) = 1.016, 95% CI 1.003–1.032), courses of postnatal antibiotics (aOR =1.103, 95% CI 1.046–1.168), incidence of postnatal ear infection (aOR = 1.137, 95% CI 1.046–1.236), and decreased duration of breastfeeding (aOR 0.948, 95% CI 0.932–0.965) are all associated with ASD when adjusted for eight demographic variables. A weak association between oral vitamin D drop exposure and ASD was also found when adjusted for breastfeeding and demographics (aOR 1.025, 95% CI 0.995–1.056).

[419] Bittker et al. Acetaminophen, antibiotics, ear infection, breastfeeding, vitamin D drops, and autism: an epidemiological study. Neuropsychiatr Dis Treat. 2018 May 31;14:1399-1414. doi: 10.2147/NDT.S158811. PMID: 29910617; PMCID: PMC5987866.

In conclusion, this study adds to evidence that postnatal acetaminophen use, postnatal antibiotic use, incidence of ear infection, and early weaning are associated with an increased risk of ASD. It also finds that postnatal oral vitamin D drops are weakly associated with ASD when adjusted for breastfeeding and demographics.

Liew 2016c

The study is a prospective cohort study.[420]

Information used to determine exposure: The exposure of interest was acetaminophen use during pregnancy, which was assessed using three computer-assisted telephone interviews.

Outcome definition and determination: The outcome of interest was a diagnosis of ASD in the offspring, which was determined using records from the Danish hospital and psychiatric registries.

Control group: The control group consisted of children whose mothers did not report using acetaminophen during pregnancy.

Study size: The study included 64,322 children and mothers enrolled in the Danish National Birth Cohort (DNBC; 1996-2002) for an average of 12.7 years.

Confounding factors or biases and how or if they were controlled: The study controlled for several potential confounding factors, including maternal age, education, smoking during pregnancy, pre-pregnancy body mass index (BMI), parity, and hyperkinetic disorders. The authors used Cox proportional hazards models to estimate hazard ratios (HRs) and 95% confidence intervals (CIs).

Limitations: Self-reported data from mothers, residual confounding by indication, or genetic factors.

Results: In this study, maternal use of APAP was associated with ASD by ever versus never use (aHR = 1.19, 95% CI 1.04-1.35), APAP use during all three trimesters (aHR = 1.39, 95% CI 1.14–1.70), and >20 weeks APAP use during pregnancy (aHR = 1.39, 95% CI 1.14–1.70). For infantile autism, often considered more severe, was associated with APAP use during all three trimesters (aHR = 1.49, 95% CI 1.07–2.07) and >20 weeks APAP use during pregnancy (aHR = 1.62, 95% CI 1.05–2.51). Inclusion of ASD or infantile autism with hyperkinetic symptoms was also reported to increase risk (Figure 32).

[420] Liew et al. Maternal use of acetaminophen during pregnancy and risk of autism spectrum disorders in childhood: A Danish national birth cohort study. Autism Res. 2016 Sep;9(9):951-8. doi: 10.1002/aur.1591. Epub 2015 Dec 21. PMID: 26688372.

	Autism spectrum disorders				Infantile autism			
	Hyperkinetic −		Hyperkinetic +		Hyperkinetic −		Hyperkinetic +	
	No. cases	Adjusted HR[a] (95% CI)	No. cases	Adjusted HR[a] (95% CI)	No. cases	Adjusted HR[a] (95% CI)	No. cases	Adjusted HR[a] (95% CI)
Acetaminophen use during pregnancy								
Never used	295	1.00 (ref)	106	1.00 (ref)	110	1.00 (ref)	29	1.00 (ref)
Ever used	412	1.07 (0.92–1.24)	214	1.51 (1.19–1.92)	147	0.98 (0.77–1.26)	59	1.55 (0.98–2.45)
All three trimesters	87	1.25 (0.98–1.60)	46	1.77 (1.24–2.53)	33	1.23 (0.83–1.83)	17	2.45 (1.32–4.53)
Weeks of acetaminophen use throughout pregnancy								
0 weeks	295	1.00 (ref)	106	1.00 (ref)	110	1.00 (ref)	29	1.00 (ref)
1–5 week	203	1.06 (0.88–1.27)	105	1.51 (1.14–2.00)	73	1.00 (0.74–1.34)	25	1.32 (0.75–2.31)
6–20 weeks	50	0.99 (0.73–1.34)	31	1.62 (1.08–2.45)	15	0.78 (0.46–1.34)	8	1.55 (0.68–3.50)
>20 weeks	44	1.42 (1.02–1.97)	22	1.89 (1.19–3.02)	17	1.45 (0.86–2.43)	7	2.25 (1.00–5.07)
		P-trend = 0.074		P-trend = 0.019		P-trend = 0.233		P-trend = 0.071

Figure 32. Hazard Ratios for ASD and Infantile Autism with Hyperkinetic Symptoms. Adjusted child's sex, birth year, maternal age at birth, parity, socioeconomic status, maternal smoking and alcohol drinking during pregnancy, maternal pre-pregnancy body mass index, folic acid intake during pregnancy, mother's psychiatric illnesses, maternal diseases in muscles/joints, fever, or infection/inflammation during pregnancy, maternal use of ibuprofen and aspirin during pregnancy. (Liew et al 2016c)

Overall, the study found that prenatal use of acetaminophen was associated with an increased risk of ASD accompanied by hyperkinetic symptoms but not with other ASD cases. Longer duration of use (i.e., use for >20 weeks in gestation) increased the risk of ASD or infantile autism with hyperkinetic symptoms almost twofold.

Saunders 2019 (~)

The study is a case-control study that investigated the association between prenatal exposures and the risk of autism spectrum disorder (ASD) in children.[421]

Information used to determine exposure: The exposure of interest was prenatal exposures, which were assessed using a questionnaire that asked about medication use, smoking, alcohol consumption, and other environmental exposures during pregnancy.

Outcome definition and determination: The outcome of interest was a diagnosis of ASD in children, which was determined using the Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition (DSM-5) criteria.

Control group: The control group consisted of children without ASD who were matched to cases by age and sex.

Study size: The study included 215 mothers of children (107 with ASD and 108 without ASD).

Confounding factors or biases and how or if they were controlled: The study controlled for several potential confounding factors, including maternal age, education, income, and pre-pregnancy body mass index (BMI). The authors used logistic regression models to estimate odds ratios (ORs) and 95% confidence intervals (CIs).

Limitations: Limitations included being a self-reported questionnaire from mothers, which may have reduced accuracy due to poor recall or biased recall. For example, none of the participants indicated

[421] Saunders et al. A Comparison of Prenatal Exposures in Children with and without a Diagnosis of Autism Spectrum Disorder. Cureus. 2019 Jul 24;11(7):e5223. doi: 10.7759/cureus.5223. PMID: 31565625; PMCID: PMC6758968.

alcohol consumption, although some participants admitted to using illicit drugs. Another limitation is that the study had a large number of variables and smaller sample sizes per group, which made it difficult to assess statistically the number of cigarettes smoked, types of medications used, and the formulation of the prenatal vitamin. Additionally, socioeconomic status (SES) was not controlled for in the study.

Results: The authors reported that 46.7% of mothers of children with ASD and 48.3% of mothers of children without ASD reported using APAP during pregnancy. Significant associations (Chi-square test) between multiple variables, including sibling with ASD, family member with ASD, medication use, and cigarette use. Significant odds ratios were reported for ASD family (OR 2.72, 95% CI 1.29-5.73), use of other medications (OR 2.29, 95% CI 1.21-4.36), and smoking (OR 2.56, 95% CI 1.91-5.49).

Overall, the study found that there was no statistically significant association found between APAP use during pregnancy and a later diagnosis of ASD in children. The lack of adjustment by SES was not controlled for in the study. Other studies have reported that high SES is associated with APAP use, improved developmental indices and inversely with smoking. In the absence of controlling for SES, the authors indicate, "Without controlling for these missing pieces, the current model cannot fully account for all variables in the complex diagnosis of ASD."

Ji 2020

This is a prospective cohort study design using samples selected from the Boston Birth Cohort.[422]

Information used to determine exposure: Information about acetaminophen exposure was based on cord plasma biomarkers of fetal exposure to acetaminophen.

Outcome definition and determination: The outcomes of interest were childhood attention-deficit/hyperactivity disorder and autism spectrum disorder. Diagnoses were obtained from electronic medical record review.

Control group: The control group consisted of children without attention-deficit/hyperactivity disorder or autism spectrum disorder diagnosis.

Study size: The study included a total of 996 mother-infant dyads.

Confounding factors or biases and how or if they were controlled: Potential covariates such as maternal age, race/ethnicity, education, marital status, pre-pregnancy body mass index, smoking during pregnancy, alcohol use during pregnancy, and child sex were included in the statistical model.

Limitations: The study excluded confounding by indication but was unable to exclude the potential residual confounders of genetic and environmental factors.

Results: The highest concentrations of APAP in cord blood were associated with ASD (aOR = 2.88, 95% CI 1.80-4.66), ADHD (aOR = 3.72, 95% CI 1.70-8.55), and ASD and ADHD (aOR = 3.38, 95% CI 1.25-

[422] Ji et al. Association of Cord Plasma Biomarkers of In Utero Acetaminophen Exposure With Risk of Attention-Deficit/Hyperactivity Disorder and Autism Spectrum Disorder in Childhood. JAMA Psychiatry. 2020 Feb 1;77(2):180-189. doi: 10.1001/jamapsychiatry.2019.3259. PMID: 31664451; PMCID: PMC6822099.

9.85). Other developmental disorders were not associated with APAP concentrations (aOR = 0.86, 95% CI 0.56-1.31).

Overall, the study reported that cord plasma biomarkers of fetal exposure to acetaminophen were associated with significantly increased risk of childhood attention-deficit/hyperactivity disorder and autism spectrum disorder. The study concludes that in utero exposure to acetaminophen is associated with increased risk of attention-deficit/hyperactivity disorder and autism spectrum disorder in children and warrant additional investigations. Maternal APAP biomarkers were also associated with increased risk of ADHD diagnosis in offspring.

Carey 2022

The study examined the associations between maternal biomarkers of oxidative stress (OS), measured during pregnancy, and child ASD-related outcomes.[423] The biomarkers of interest included: 8-oxo-deoxyguanine (8-OHdG), an oxidative DNA adduct; Glutathione (GSH), the primary intracellular ROS antioxidant and detoxification mechanism in the body; Glutathione disulfide (GSSG), the oxidized counterpart to GSH and a marker of extra-cellular oxidative stress; the ratio of GSH:GSSG, which acts as an indicator of redox balance; and 3-nitrotyrosine (nitrotyrosine), an amino acid modified by nitrosylation that is formed under oxidative/nitrosative stress.

Outcome definition and determination: ASD-related outcomes were assessed via the Social Responsiveness Scale (SRS), Mullen Scales of Early Learning (MSEL-ELC), and ASD clinical diagnosis at 36 months. The SRS is a questionnaire designed to assess a child's reciprocal social behaviors in social interactions. The MSEL assesses early intellectual development and school readiness that is strongly correlated with intellectual quotient (IQ) scores. ASD diagnosis at 36 months was determined by clinical evaluation, which included the MSEL and the Autism Diagnostic Observation Schedule (ADOS).

Control group: This study did not have a traditional control group as it was a cohort study following pregnancies among women who already had an autistic child.

Study size: In total, 173 participants were eligible based on the outlined criteria however, the total number of participants included in final analyses varied by outcome of interest, from 149 to 164 participants.

Confounding factors or biases and how or if they were controlled: Covariates included in analyses were selected based on a priori knowledge, as well as statistical relevance. The primary models were adjusted for maternal age, race/ethnicity, maternal pre-pregnancy body mass index (BMI), child's sex, and prenatal vitamin use in the first month of pregnancy. Additional covariates considered in sensitivity analyses included pregnancy complications, smoking status, gestational age at DNA/plasma sampling, antioxidant intake, and use of folic acid supplementation in the first month of pregnancy.

Limitations: Reported limitations included limited timing, and familial background risk could change the nature of associations.

[423] Carey et al. Examining associations between prenatal biomarkers of oxidative stress and ASD-related outcomes using quantile regression. J Autism Dev Disord. 2022 Jun 9:10.1007/s10803-022-05625-9. doi: 10.1007/s10803-022-05625-9. Epub ahead of print. PMID: 35678944; PMCID: PMC9732143.

Results: Increasing GSH:GSSG was associated with minor increases in SRS scores (50th percentile β: 1.78, 95% CI: 0.67, 3.06). Significant crude ORs for oxidized glutathione and ASD (GSSG, OR 2.27, 95% 1.07, 4.81) were not significant after adjustments (aOR = 2.03, 95% CI 0.89, 4.62).

The conclusion of the study is that there was not a strong relationship between the biomarkers of oxidative stress (OS) measured in maternal samples from mid- to late-pregnancy and child ASD-related outcomes measured at 36 months. However, there was evidence that greater antioxidant balance (higher GSH:GSSG ratio) was inversely associated with risk of ASD and non-typical development according to diagnostic assessments.

Weight of the Evidence for the Association of APAP with ASD

Overall, **these studies provide "some evidence" that APAP use during pregnancy causes developmental toxicity in offspring**, this conclusion is based on study limitations and the reported association between APAP use during pregnancy and ASD outcomes in offspring.

Based on the totality of reviewed studies indicated above and summarized below, the weight of the evidence indicates **there is a moderate association of APAP exposure during pregnancy with ASD or related outcomes in children**. Regarding dose, "therapeutic" dosages are sufficient to cause harm, with higher concentrations being associated with higher risk, aOR = 2.88 (95% CI 1.80-4.66) , see Ji 2020. Regarding duration, a longer duration increases risk, with an aHR = 1.23 (95% CI 1.02-1.48) with 2-5 weeks of exposure, see Liew 2016, and a 1.49-2.01 increased risk reported for exposure throughout pregnancy, summarized below. There are multiple studies and lines of evidence that support the association, but some studies did not detect an association. Factors that support and affect the weight of the evidence include:

- The study designs varied from cohort studies, case-control studies, to internet-based surveys, which have different strengths and limitations in terms of validity, reliability, and generalizability.
- The exposure and outcome assessments vary from self-report, medical records, biomarkers, to standardized tests, which have different sources of error, bias, and confounding.
- The control groups vary from children without ASD or related outcomes, children without APAP exposure, to children with other developmental disorders, which have different implications for comparison and interpretation.
- The confounding factors vary from maternal age, education, smoking, BMI, to genetic and environmental factors, which have different effects on the association and may not be fully controlled for in all studies.

The range of odds ratios support the association between APAP exposure during pregnancy or childhood and ASD are reported below, with multiple studies supporting significant positive associations. This means that APAP exposure during pregnancy or childhood increases the risk of ASD or related outcomes in children compared to no exposure. The data from these studies are summarized below:

Study	Exposure	Outcome	Findings
Avella-Garcia 2016	Prenatal APAP exposure	Autism spectrum symptoms in males	1.41 (95% CI 1.01–1.98) IRR
Avella-Garcia 2016	Prenatal APAP exposure	Commission errors in attention function	1.10 (95% CI 1.03–1.17) IRR
Bittker 2018	Postnatal APAP exposure	ASD	1.016 (95% CI 1.003–1.032) aOR
Liew 2016c	Prenatal APAP exposure	ASD	1.19 (95% CI 1.04-1.35) for ever versus never use; 1.39 (95% CI 1.14–1.70) for use during all three trimesters; 1.39 (95% CI 1.14–1.70) for >20 weeks use, aHR
Liew 2016c	Prenatal APAP exposure	infantile autism	1.49 (95% CI 1.07–2.07) for use during all three trimesters; 1.62 (95% CI 1.05–2.51) for >20 weeks use, aHR
Liew 2016c	Prenatal APAP exposure	ASD or infantile autism with HKS	ASD: 1.51 (95% CI 1.19–1.92) for ever versus never use; 1.77 (1.24–2.53) for use during all three trimesters, aHR; 1.51 (95% CI 1.14–2.00), 1.62 (95% CI 1.08–2.45), 1.89 (95% CI 1.19–3.02) for 1-5, 6-20, >20 weeks, respectively Infantile: 2.45 (95% CI 1.32-4.53) for use during all three trimesters; 2.25 (95% CI 1.00-5.07) for >20 weeks use, aHR
Ji 2020	Cord plasma biomarkers	ASD	2.88 (95% CI 1.80-4.66) for the highest concentration group, aOR
Ji 2020	Cord plasma biomarkers	ADHD	3.72 (95% CI 1.70-8.55) for the highest concentration group, aOR
Ji 2020	Cord plasma biomarkers	ASD and ADHD	3.38 (95% CI 1.25-9.85) for the highest concentration group, aOR

Avella-Garcia 2016:

- Prenatal APAP exposure was associated with more autism spectrum symptoms in males (incidence rate ratio (IRR) = 1.41, 95% CI 1.01–1.98) and adverse effects on attention function in both genders (IRR = 1.10, 95% CI 1.03–1.17 for commission errors; β = 0.75, 95% CI 0.13–0.02 for detectability scores).

Bittker 2018:

- Postnatal APAP exposure was associated with ASD (adjusted odds ratio (aOR) = 1.016, 95% CI 1.003–1.032), along with postnatal antibiotic use (aOR =1.103, 95% CI 1.046–1.168), incidence of ear infection (aOR = 1.137, 95% CI 1.046–1.236), and early weaning (aOR 0.948, 95% CI 0.932–0.965). Oral vitamin D drop exposure was weakly associated with ASD (aOR 1.025, 95% CI 0.995–1.056).

Liew 2016c:

- Prenatal APAP exposure was associated with ASD (adjusted hazard ratio (aHR) = 1.19, 95% CI 1.04-1.35 for ever versus never use; aHR = 1.39, 95% CI 1.14–1.70 for use during all three trimesters; aHR = 1.39, 95% CI 1.14–1.70 for >20 weeks use). Longer duration of use increased the risk of ASD or infantile autism with hyperkinetic symptoms almost twofold (aHR = 1.49, 95% CI 1.07–2.07 for use during all three trimesters; aHR = 1.62, 95% CI 1.05–2.51 for >20 weeks use).

- Prenatal APAP exposure was associated with ASD accompanied by hyperkinetic symptoms (adjusted hazard ratio (aHR) = 1.51, 95% CI 1.19–1.92 for ever versus never use; aHR = 1.77, 95% CI 1.24–2.53 for use during all three trimesters; aHR = 1.89, 95% CI 1.19–3.02 for >20 weeks use). Longer duration of use increased the risk of infantile autism accompanied by hyperkinetic symptoms almost twofold (aRR = 2.45, 95% CI 1.32–4.53 for use during all three trimesters; aRR = 2.25, 95% CI 1.00–5.07 for >20 weeks use).

Ji 2020:

- The study found that the highest concentrations of APAP in cord blood were associated with ASD (aOR = 2.88, 95% CI 1.80-4.66), ADHD (aOR = 3.72, 95% CI 1.70-8.55), and ASD and ADHD (aOR = 3.38, 95% CI 1.25-9.85). Other developmental disorders were not associated with APAP concentrations (aOR = 0.86, 95% CI 0.56-1.31).

Carey 2022:

- Increasing GSH:GSSG was associated with minor increases in SRS scores (50th percentile β: 1.78, 95% CI: 0.67, 3.06). Significant crude ORs for oxidized glutathione and ASD (GSSG, OR 2.27, 95% 1.07, 4.81) were not significant after adjustments (aOR = 2.03, 95% CI 0.89, 4.62).

B. Studies Examining the Association of APAP with ADHD

Anand 2021

This study used the Boston Birth Cohort (BBC), a prospective birth cohort study of 3165 mother-child pairs followed at the Boston Medical Center (BMC) from 1998 to 2018.[424]

Outcome definition and determination: Childhood neurodevelopmental diagnoses were categorized based on clinician documentation of primary and secondary diagnoses in electronic medical records up to June 2018. The ADHD-only category included children with only ADHD-related codes (ICD-9 codes 314.0–314.9; ICD-10 codes F90.0–F90.9) and excluded children with autism spectrum disorder (ASD) diagnoses (ICD-9 codes 299.0-299.91; ICD-10 codes F84.0–F84.9) or other mental, behavioral, and neurodevelopmental disorders (ICD-9 codes 290–319; ICD-10 codes F01–F99).

[424] Anand et al. Perinatal Acetaminophen Exposure and Childhood Attention-Deficit/Hyperactivity Disorder (ADHD): Exploring the Role of Umbilical Cord Plasma Metabolites in Oxidative Stress Pathways. Brain Sci. 2021 Sep 30;11(10):1302. doi: 10.3390/brainsci11101302. PMID: 34679367; PMCID: PMC8533963.

Control group: The neurotypical development category included children without ADHD, ASD, or other developmental disability diagnoses.

Study size: The source population of 3165 mother-child dyads included 433 (13.7%) participants with a childhood diagnosis of ADHD only and 1444 (45.6%) with neurotypical development. Of these, 965 participants had cord plasma analyte data when excluding siblings, of which 248 (25.7%) had childhood ADHD and 320 (33.2%) had neurotypical development.

Confounding factors or biases and how or if they were controlled: The study included several covariates drawing upon previously published analyses of the BBC such as maternal age at delivery, parity, maternal race/ethnicity, maternal education level, maternal BMI, stress during pregnancy, smoking during pregnancy, alcohol use before or during pregnancy, marital status, child sex, delivery type, preterm birth, low birthweight, stress during pregnancy, and maternal fever during pregnancy.

Limitations: The study examined cord blood, removing recall bias, but limiting temporal exposure to late gestation. Other limitations include unmeasured, residual confounding, and genetics.

Results: Cord unmetabolized APAP >50th percentile was associated with higher odds of ADHD diagnosis (aOR: 2.10, 95% CI 1.43, 3.11). In adjusted regressions, increasing levels of cord methionine (aOR: 1.43, 95% CI 1.17, 1.77), serine (aOR: 1.38, 95% CI 1.14, 1.68), glycine (aOR: 1.38, 95% CI 1.14, 1.68), and 8-hydroxy-deoxyguanosine (aOR: 1.24, 95% CI 1.01, 1.52) were associated with higher odds of ADHD.

Overall, the study concludes APAP was associated with increased risk of ADHD. The amino acids involved in producing the antioxidant glutathione, including methionine, serine, and glycine, were also associated with increased odds of ADHD. Additionally, DNA oxidation (8-hydroxy-deoxyguanosine), a marker of oxidative damage, was also linked to ADHD.

Baker 2020

This was a prospective cohort study.[425]

Information used to determine exposure: Prenatal APAP exposure was measured in meconium.

Outcome definition and determination: The outcome was ADHD in children aged 6 to 7 years, as determined by the Conners' Parent Rating Scale-Revised: Long Form.

Control group: The control group consisted of children who were not exposed to acetaminophen prenatally.

Study size: The study included 394 eligible children with 345 meconium samples collected at birth.

Confounding factors or biases and how or if they were controlled: The study controlled for several potential confounding factors, including maternal age, education, smoking during pregnancy, alcohol consumption during pregnancy, and child sex.

[425] Baker et al. Association of Prenatal Acetaminophen Exposure Measured in Meconium With Risk of Attention-Deficit/Hyperactivity Disorder Mediated by Frontoparietal Network Brain Connectivity. JAMA Pediatr. 2020 Nov 1;174(11):1073-1081. doi: 10.1001/jamapediatrics.2020.3080. PMID: 32986124; PMCID: PMC7522774.

Limitations: The study had several limitations, including the potential for residual confounding and the possibility that meconium may not accurately reflect self-reported prenatal acetaminophen exposure. Meconium can identify drug exposure in the 2nd and 3rd trimester and reveal the relative concentration compared to others in the cohort, but cannot by itself identify exact dose or duration.

Results: Detection of APAP in meconium was associated with increased odds of ADHD (OR = 2.43, 95% CI, 1.41-4.21). A dose-response association was detected; each doubling of exposure increased the odds of ADHD by 10% (OR = 1.10, 95% CI 1.02-1.19).

Overall, the study found that children exposed to acetaminophen prenatally were at increased risk of ADHD at ages 6 to 7 years. Prenatal acetaminophen exposure was also associated with increased negative connectivity between the left prefrontal cortex and the right precentral/frontal gyrus, which mediated the association of acetaminophen with hyperactivity.

Chen 2019

The study is a nationwide case-control study that investigated the association between prenatal exposure to acetaminophen and the risk of attention-deficit/hyperactivity disorder (ADHD) in children.[426]

Information used to determine exposure: The exposure of interest was prenatal exposure to acetaminophen, which was assessed using data from Taiwan's National Health Insurance Research Database.

Outcome definition and determination: The outcome of interest was a diagnosis of ADHD in children, which was determined using International Classification of Diseases, Ninth Revision (ICD-9) codes from national registers.

Control group: The control group consisted of children without ADHD who were matched to cases by age and sex.

Study size: The study included 950 mother children pairs with ADHD and 3,800 control pairs.

Confounding factors or biases and how or if they were controlled: The study controlled for several potential confounding factors, including maternal age at delivery, parity, maternal smoking during pregnancy, maternal pre-pregnancy body mass index (BMI), maternal education, maternal psychiatric history, and family income. The authors used conditional logistic regression models to estimate odds ratios (ORs) and 95% confidence intervals (CIs).

Limitations: Proposed limitations included underestimated prevalence of ADHD and potential misclassification of controls if some had ADHD, which would bias towards the null hypothesis. Self-administration of APAP may have occurred if it was purchased over-the-counter, as use was based on prescriptions. Potential interactions with other medications, bias confounding. There was also potential misclassification of mothers, because no mothers were diagnosed with ADHD or substance use disorders.

Results: APAP exposure in the second trimester (OR = 1.19, 95% CI 1.00-1.40), first and second trimester (OR = 1.28, 95% CI 1.00-1.64), or any trimester (OR = 1.20, 95% CI 1.01-1.42) was associated with an increased risk of ADHD in offspring. Associations remained after excluding gestational infections and

[426] Chen et al. Prenatal Exposure to Acetaminophen and the Risk of Attention-Deficit/Hyperactivity Disorder: A Nationwide Study in Taiwan. J Clin Psychiatry. 2019 Sep 10;80(5):18m12612. doi: 10.4088/JCP.18m12612. PMID: 31509360.

maternal health disorders (OR = 1.33, 95% CI 1.04-1.69)(OR = 1.68, 95% CI 1.18-2.40)(OR = 1.40, 95% CI 1.14-1.73), respectively.

Overall, the study concluded that prenatal exposure to APAP is associated with an increased risk of ADHD in children.

Ji 2018

This is a prospective cohort study design using samples selected from the Boston Birth Cohort.[427]

Information used to determine exposure: Information about APAP exposure was based on maternal biomarkers of APAP intake measured in plasma samples obtained within 1–3 days postpartum.

Outcome definition and determination: The outcome of interest was ADHD diagnosis in offspring. ADHD diagnoses were obtained from electronic medical record review.

Control group: The control group consisted of children without ADHD diagnosis.

Study size: The study included a total of 1180 children, including 188 with ADHD diagnosis.

Confounding factors or biases and how or if they were controlled: Potential covariates such as maternal age, race/ethnicity, education, marital status, pre-pregnancy body mass index, smoking during pregnancy, alcohol use during pregnancy, and child sex were included in the statistical model.

Limitations: The study only measured maternal APAP metabolite levels once, shortly after childbirth, which reflects recent use but cannot provide detailed information on dosage and usage patterns during specific time periods. The measurement method did not include acetaminophen sulfate, a significant metabolite, but estimates of total APAP burden were derived from other measured metabolites. Potential confounding by familial factors and unmeasured or unknown confounders, despite adjustments and propensity score analyses. The study sample predominantly consisted of urban low-income minority populations, limiting the generalizability of the results to all pregnant women in the U.S.

Results: Below median and above median levels of maternal acetaminophen burden were associated with a 58% and 88% increase in the odds of ADHD diagnosis, respectively (Model 6: OR for below median = 1.58, 95% CI 1.02-2.46; OR for above median 1.88, 95% CI 1.18, 3.00). However, there were no significant associations found between maternal plasma levels of acetaminophen metabolites and the risks of ASD diagnosis and other DD diagnoses.

The study concludes that maternal plasma biomarkers of APAP use shortly after delivery were associated specifically with an increased risk of ADHD diagnosis in offspring. No associations were found with other developmental disorders. These findings remained significant even after adjusting for known confounders and indications for APAP use.

[427] Ji et al. Maternal Biomarkers of Acetaminophen Use and Offspring Attention Deficit Hyperactivity Disorder. Brain Sci. 2018 Jul 3;8(7):127. doi: 10.3390/brainsci8070127. PMID: 29970852; PMCID: PMC6071105.

Gustavson 2021

This study is a longitudinal sibling control study that examined the association between APAP use during pregnancy and offspring ADHD.[428] The sibling control model, also known as the sibling comparison design or within-family design, is a research approach used in epidemiological and genetic studies to compare outcomes between siblings within the same family.

Exposure: The exposure of interest in this study was maternal APAP use during pregnancy. Information on APAP use was obtained from maternal self-reported questionnaires. This is a study limitation, because self-reported maternal APAP use during pregnancy may be subject to recall bias or measurement error.

Outcome definition and determination: The outcome of interest in this study was ADHD diagnosis in the offspring. ADHD diagnoses were obtained from the Norwegian Patient Registry.

Control group: This study used a sibling control design, where siblings discordant for prenatal APAP exposure were compared regarding risk of having an ADHD diagnosis.

Study size: The study included 26,613 children from 12,902 families participating in the prospective Norwegian Mother, Father, and Child Cohort Study (MoBa).

Confounding factors or biases and how or if they were controlled: The study adjusted for several potential confounding factors, including maternal age, parity, child's sex, maternal education level, smoking status, alcohol use, and symptoms of depression and anxiety. Additionally, the study used propensity score matching to control for potential confounding by indication.

Limitations: Only discordant siblings contribute informative data in the sibling design. In this study, 623 mothers participated with children discordant on outcome, 306 mother participated with children that were discordant on exposure as well as outcome. 380 mothers participated with children discordant on the exposure for 29 days or more, and only 34 of these had children discordant on outcome. The authors indicate that the "statistical power to detect within effects was relatively low."

While the sibling control design allows for the control of unmeasured familial confounding factors, it may also control for potential mediating factors that affect all siblings, even if only one is exposed. This may lead to underestimation of association estimates. Sibling control models do not control for confounders that are not shared between siblings, and this can also introduce bias in the study.

Having an initial child with ADHD may affect the decision and timing of having more children, and this may have biased the current results. The order of the siblings was not reported to examine this impact. The sibling order has been reported to have an impact on diagnosis of ADHD or ASD in a sibling. For example, a separate study supported a relatively greater impact of within-diagnosis sibling recurrence risk and sibling cross-aggregation of ADHD and ASD among later-born siblings of children with either disorder.[429] Specifically, the study found that later-born siblings of children with ASD or ADHD appear

[428] Gustavson et al. 2021. Acetaminophen use during pregnancy and offspring attention deficit hyperactivity disorder – a longitudinal sibling control study. JCPP Advances. 2021;e12020. DOI: 10.1002/jcv2.12020

[429] Carey et al. Examining associations between prenatal biomarkers of oxidative stress and ASD-related outcomes using quantile regression. J Autism Dev Disord. 2022 Jun 9:10.1007/s10803-022-05625-9. doi: 10.1007/s10803-022-05625-9. Epub ahead of print. PMID: 35678944; PMCID: PMC9732143.

to be at elevated risk for the same disorder and of being diagnosed with the other disorder. Compared with later-born siblings of children without ADHD or ASD, later-born siblings of children with ASD were more likely to be diagnosed with ASD (odds ratio (OR), 30.38; 95% CI, 17.73-52.06) or ADHD in the absence of ASD (OR, 3.70; 95% CI, 1.67-8.21). Compared with later-born siblings of children without a diagnosis, later-born siblings of children with ADHD were more likely to be diagnosed with ADHD (OR, 13.05; 95% CI, 9.86-17.27) or ASD in the absence of ADHD (OR, 4.35; 95% CI, 2.43-7.79). These findings indicate that having a sibling previously diagnosed with ADHD or ASD increased the risk more for the second child.

Results: This study found that children exposed to acetaminophen up to 28 days during pregnancy did not have an increased risk of receiving an ADHD diagnosis compared to unexposed children. However, long-term exposure (29 days or more) was associated with a two-fold increase in risk of ADHD diagnosis (adjusted hazard ratio (aHR) = 2.02, 95% CI = 1.17–3.25). All children (both exposed and unexposed) born to a mother with long-term use of acetaminophen in one pregnancy, had increased risk of receiving an ADHD diagnosis compared to children of mothers who did not use acetaminophen in any pregnancy (aHR = 2.77, 995% CI = 1.48–5.05). In the sibling control model, the association between long-term acetaminophen use and ADHD in the child was no longer present (aHR = 1.06, 995% CI = 0.51–2.05). This indicates that the observed association between long-term acetaminophen use during pregnancy and ADHD in the child may at least partly be confounded by unobserved family factors.

The study reported that both the exposed and the unexposed children of mothers with long-term use of APAP in one of the pregnancies had increased risk of receiving an ADHD diagnosis. This indicates that the observed association between long-term acetaminophen use during pregnancy and ADHD in the child may at least partly be confounded by unobserved family factors.

Liew 2014

Study type: The study was a prospective observational cohort study.[430]

Information used to determine exposure: Acetaminophen use during pregnancy was assessed via 3 computer-assisted telephone interviews during pregnancy and 6 months after childbirth.

Outcome definition and determination: The main outcomes measured were the risk of receiving an hyperkinetic disorder (HKD) diagnosis, using ADHD medications, or having ADHD-like behaviors at age 7 years after prenatal exposure to acetaminophen. Behavioral outcomes were determined Strengths and Difficulties Questionnaire (SKD).

Study size: The study included 64,322 live-born children and mothers enrolled in the Danish National Birth Cohort during 1996-2002 for the HKD analysis. They further excluded children whose caregiver did not respond to a self-administered online/mail questionnaire when the child turned 7 years, resulting in 40,916 for children for the SKD.

Confounding factors or biases and how or if they were controlled: To address potential confounding by indication, they used stratified analyses according to self-reported episodes of fever, inflammation, or infection during pregnancy. Other potential confounders included the birth year of the child, birth weight,

[430] Liew et al. Acetaminophen use during pregnancy, behavioral problems, and hyperkinetic disorders. JAMA Pediatr. 2014 Apr;168(4):313-20. doi: 10.1001/jamapediatrics.2013.4914. PMID: 24566677.

and sex, as well as maternal age, parity, gestational age at delivery, socioeconomic status, smoking and alcohol consumption during pregnancy, prepregnant body mass index, and maternal self-reported psychiatric illnesses. Women were asked to self-report if they had psychiatric illnesses and had sought medical or psychological help for depression, anxiety, childhood psychiatric disorder, family problems/life crisis, or other mental health problems. Parents provided self-reported childhood behavior problems (SDQ scale) were considered for analyzing ADHD-like behaviors in children. Other potential confounders such as age of father at the birth of the child, Apgar scores, and season of conception were evaluated but did not significantly affect the effect estimates (<1% change) and were not included in the final models.

Limitations: The inability to accurately assess dosage or number of pills taken and the lack of specific information on the gestational week of APAP use affected the precision of effect estimation. However, the authors reported that excluding these cases when assessing exposure duration did not alter the results or conclusions. Flawed recall of drug names, frequency, and timing of use is likely to be nondifferential in relation to the disease status of the child, resulting in bias towards the null and underestimated effects.

Results: Maternal acetaminophen use during pregnancy was associated with a higher risk of children receiving a hospital diagnosis of HKD (hazard ratio = 1.37; 95% CI, 1.19-1.59), use of ADHD medications (hazard ratio = 1.29; 95% CI, 1.15-1.44), or having ADHD-like behaviors at age 7 years (risk ratio = 1.13; 95% CI, 1.01-1.27). The associations were stronger with use in multiple trimesters and showed an exposure-response trend with increasing frequency of acetaminophen use during gestation. The results were not confounded by maternal inflammation, infection during pregnancy, the mother's mental health problems, or other potential confounders examined.

The authors conclude that prenatal exposure to APAP increased the risk of children receiving a hospital diagnosis of HKD or ADHD medication and exhibiting ADHD-like behaviors. The risk increased with higher frequency of APAP use in an exposure-response manner. These associations support APAP as a contributor to the increasing incidence of HKD/ADHD.

Liew 2016b

This is a prospective cohort study that followed mothers and children from pregnancy to age 5.[431]

Information used to determine exposure: Exposure to paracetamol was determined by self-reported use in three telephone interviews conducted at gestational weeks 12 and 30 and 6 months postpartum.

Outcome definition and determination: The outcomes were attention and executive function in children at age 5, measured by the Test of Everyday Attention for Children at Five (TEACh-5) and the Behaviour Rating Inventory of Executive Function (BRIEF), completed by trained psychologists, parents and preschool teachers.

Control group: The control group consisted of mothers who never used paracetamol during pregnancy.

[431] Liew et al. Paracetamol use during pregnancy and attention and executive function in offspring at age 5 years. Int J Epidemiol. 2016 Dec 1;45(6):2009-2017. doi: 10.1093/ije/dyw296. PMID: 28031314.

Study size: The study included 1491 mother-child pairs who completed all three telephone interviews and participated in the neuropsychological assessment at age 5.

Confounding factors or biases and how or if they were controlled: The study adjusted for potential confounders such as parental education, maternal IQ, maternal mental health, prenatal smoking and drinking, parity, maternal age, child's sex, maternal pre-pregnancy BMI, maternal musculoskeletal diseases, fever or infection/inflammation during pregnancy, and maternal use of ibuprofen or aspirin. The study also used inverse probability weights to account for subject selection and non-participation. However, the study acknowledged that residual confounding or unmeasured confounding may still exist, such as genetic factors, environmental exposures, or other medications.

Limitations: The study had several limitations, such as the reliance on self-reported paracetamol use, the lack of information on dose and timing of exposure, the possibility of recall bias or misclassification of exposure, the potential influence of reverse causation or indication bias, the generalizability of the findings to other populations or settings, and the uncertainty of the biological mechanisms underlying the observed associations.

Results: First-trimester APAP use was associated with poorer attention scores in childhood (mean difference -0.34, 95% CI -0.63, -0.05 for overall attention; -0.25, 95% CI -0.50, 0.01 for selective attention). Prenatal exposure to APAPl increased the risk of subnormal overall attention (OR 1.5, 95% CI 1.0-2.5), selective attention difficulties (OR 1.5, 95% CI 1.0-2.4), and parent-rated subnormal executive function (metacognition index, OR 1.5, 95% CI 0.9-2.3). Longer duration of APAP use in pregnancy further elevated the risks for subnormal overall attention or executive function.

In conclusion, this study provides evidence of a moderate association between prenatal APAP use and subnormal attention and executive function in offspring at age 5. This study provides additional support for the interaction of *in utero* exposure of APAP on adverse neurodevelopment in children.

Liew 2019

This is a prospective cohort study design using samples selected from the Nurses' Health Study II cohort.[432]

Information used to determine exposure: Information about acetaminophen exposure was based on self-reported acetaminophen use at the time of pregnancy and during two negative control exposure periods (about 4 years before and 4 years after the pregnancy). This is a study limitation, because self-reported maternal APAP use during pregnancy may be subject to recall bias or measurement error.

Outcome definition and determination: The outcome of interest was childhood attention-deficit/hyperactivity disorder. ADHD diagnoses were obtained from maternal report.

Control group: The control group consisted of children without attention-deficit/hyperactivity disorder diagnosis.

[432] Liew et al. Use of Negative Control Exposure Analysis to Evaluate Confounding: An Example of Acetaminophen Exposure and Attention-Deficit/Hyperactivity Disorder in Nurses' Health Study II. Am J Epidemiol. 2019 Apr 1;188(4):768-775. doi: 10.1093/aje/kwy288. PMID: 30923825; PMCID: PMC6438812.

Study size: The study included a total of 8,856 children born in 1993–2005 to women enrolled in the Nurses' Health Study II cohort. ADHD was reported in 721 children, versus 8,135 without ADHD.

Confounding factors or biases and how or if they were controlled: Potential covariates such as maternal age, race/ethnicity, education, marital status, pre-pregnancy body mass index, smoking during pregnancy, alcohol use during pregnancy, and child sex were included in the statistical model.

Limitations: Reported limitations included the possibility of unmeasured confounding factors specific to pregnancy, lack of detailed information on timing and dosage of acetaminophen use, potential misclassification of exposure, inability to explore dose-response relationships, lack of data on prescription medication use as a confounder, reliance on self-reported ADHD diagnoses without clinical verification, and reliance on maternal reports, although they are generally reliable.

Results: Only APAP use at the time of pregnancy was associated with childhood ADHD (OR = 1.34, 95% CI: 1.05-1.72), and the effect estimates for the two negative control exposure periods (about 4 years before and 4 years after the pregnancy) were null.

The authors conclude that the longitudinal data from the NHS II allowed for comparisons across different time periods, supporting previous findings of a link between prenatal APAP exposure and neurodevelopmental outcomes.

Thompson 2014

This is a longitudinal cohort study design using samples selected from the Auckland Birthweight Collaborative Study.[433]

Information used to determine exposure: Information about acetaminophen exposure was based on self-reported drug use during pregnancy (acetaminophen, aspirin, antacids, and antibiotics). This is a study limitation, because self-reported maternal APAP use during pregnancy may be subject to recall bias or measurement error.

Outcome definition and determination: Symptoms of ADHD were measured at ages 7 and 11 using the parent format of the Strengths and Difficulties Questionnaire (SDQ) and also using the child format at age 11. ADHD symptoms were also measured with more specificity using the Conners' Behavioural Rating Scale: Revised (CRS:R) parent report at ages 7 and 11 years.

Control group: The control group consisted of children whose mothers did not use acetaminophen during pregnancy.

Study size: The study included a total of 871 infants of European descent sampled disproportionately for small for gestational age.

[433] Thompson et al. Associations between acetaminophen use during pregnancy and ADHD symptoms measured at ages 7 and 11 years. PLoS One. 2014 Sep 24;9(9):e108210. doi: 10.1371/journal.pone.0108210. PMID: 25251831; PMCID: PMC4177119.

Confounding factors or biases and how or if they were controlled: Potential covariates such as maternal age, education, smoking during pregnancy, alcohol use during pregnancy, and child sex were included in the statistical model.

Limitations: Study limitations included the lack of information on the dosage and trimester of APAP use during pregnancy. Unmeasured environmental factors and potential epigenetic influences may also affect disease risk and neurological outcomes. Additionally, the follow-up rate was low (59-70%), but the percentages of small for gestational age and appropriate for gestational age remained the same. The study also reported limited generalizability to European populations, and potential selection bias due to parental ADHD status should be considered.

Results: Acetaminophen was used by 49.8% of the study mothers during pregnancy. There were significantly higher total scores on all 3 SDQ formats is APAP was used during pregnancy, but there were no significant differences associated with any of the other drugs.

The authors conclude that these findings strengthen the relationship between *in utero* APAP exposure and ADHD in offspring.

Ystrom 2017

This is a cohort study using samples selected from the Norwegian Mother and Child Cohort Study and examines the association between acetaminophen exposure and ADHD diagnosis.[434]

Information used to determine exposure: The authors used self-report questionnaires from the mothers and fathers to obtain information on APAP use for each medical condition and each exposure window before and during pregnancy. They also used the Anatomic Therapeutic Chemical Classification System to classify and group medication exposure.

Outcome definition and determination: The outcome of interest was ADHD diagnosis in offspring, which was obtained from the Norwegian Patient Registry that records individual-level diagnoses from government-owned and government-financed hospitals and outpatient clinics.

Control group: The control group consisted of children who were not diagnosed with ADHD and whose parents did not use acetaminophen before or during pregnancy.

Study size: The study population included 112973 children, of whom 2246 had been diagnosed with ADHD by December 31, 2014.

Confounding factors or biases and how or if they were controlled: The authors considered several potential confounders, such as parental symptoms of ADHD, maternal alcohol use, smoking, anxiety, depression, education, marital status, BMI, age, parity, birth year, and 128 medical conditions indicative of acetaminophen use. They adjusted for these factors in their Cox proportional hazard models and stratified their analyses by each indication of use. They also used maternal preconceptional use and paternal preconceptional use as negative controls to test the specificity of the gestational effect.

[434] Ystrom et al. Prenatal Exposure to Acetaminophen and Risk of ADHD. Pediatrics. 2017 Nov;140(5):e20163840. doi: 10.1542/peds.2016-3840. PMID: 29084830; PMCID: PMC5654387.

Limitations: The authors acknowledged some limitations of their study, such as possible residual confounding by unmeasured factors (e.g., severity of indications, genetic factors), lack of validation of the ADHD diagnosis in a research clinic, and potential selection bias due to underrepresentation of young parents and smokers in the cohort.

Results: The HR for one trimester showed a non-significant association (HR = 1.07; 95% CI 0.96-1.19), while the HR for two trimesters indicated a significant association (HR = 1.22; 95% CI 1.07-1.38). Three trimesters showed a non-significant association (HR = 1.27; 95% CI 0.99-1.63). Maternal APAP use for all indications, for more than 29 days had a higher HR of 2.20 (95% CI 1.50-3.24). However, use for less than 8 days had a negative association with ADHD (HR = 0.90; 95% CI 0.81-1.00). APAP use for fever and infections, for 22 to 28 days showed a strong and significant association with ADHD (HR = 6.15; 95% CI 1.71-22.05).

The study concludes that long-term maternal APAP use during pregnancy is associated with ADHD in offspring, even after adjusting for confounders. Only use during pregnancy, not preconceptional use, showed an association with ADHD. Paternal use was also associated with the ADHD. The authors assumed this association was not biological. It appears they were unaware of the NTP (1993) study[435] and reviewed Ofirmev (IV APAP) label, see page 121 above, which indicates "increased percentage of abnormal sperm" in treated animals. Likewise, they may have also been unaware of a lab at the NIH reporting, also in November of 2017, a significant increase in % DNA fragmentation of sperm in men with high urinary APAP.[436]

Weight of the Evidence for the Association of APAP with ADHD

As indicated above, there is a considerable body of evidence supporting the association between prenatal APAP exposure and an increased risk of neurodevelopmental disorders such as ADHD and ASD in children. These studies are generally consistent in their findings, with most reporting a positive association between prenatal APAP exposure and adverse neurodevelopmental outcomes. The studies also employed various study designs, including prospective cohort studies and case-control studies, which strengthens the evidence base. However, it is important to note that most of these studies relied on maternal self-reported acetaminophen use during pregnancy, which may be subject to recall bias or measurement error. Additionally, many of these studies did not account for potential confounding factors such as underlying medical conditions that may have prompted acetaminophen use during pregnancy.

Overall, the studies above should be categorized as **"some evidence" that taking APAP during pregnancy causes developmental toxicity**, this conclusion is based upon prenatal APAP exposure and a significantly increased risk of ADHD and ASD in children.

Based on the totality of studies reviewed above and as summarized below, prenatal exposure to APAP is associated with an increased risk of ADHD or ADHD-like symptoms in children. The strength of association varies depending on the dose, duration, timing, and indication of APAP use during pregnancy. Regarding dose, "therapeutic" dosages are sufficient to cause harm. Regarding duration, a longer duration

[435] National Toxicology Program. NTP Toxicology and Carcinogenesis Studies of Acetaminophen (CAS No. 103-90-2) in F344 Rats and B6C3F1 Mice (Feed Studies). Natl Toxicol Program Tech Rep Ser. 1993 Jan;394:1-274. PMID: 12637965.
[436] Smarr et al. Male urinary paracetamol and semen quality. Andrology. 2017 Nov;5(6):1082-1088. doi: 10.1111/andr.12413. Epub 2017 Aug 29. PMID: 28853221.

increases risk, with a 2.02-6.15 fold increase in risk reported for three to four weeks of exposure. Some studies also found no significant association or a negative association between APAP exposure and ADHD outcomes, this included the sibling control model, which was reportedly underpowered by the authors. **There is a moderate association between APAP use during pregnancy and ADHD in children.** More specifically, multiple independent studies reported a significant association between APAP exposure and increased risk of ADHD or ADHD-like symptoms. The data from these studies are summarized below:

Study	Exposure	Outcome	Findings
Anand 2021	Cord blood acetaminophen >50th percentile	ADHD diagnosis	2.10 (95% CI 1.43, 3.11)
Baker 2020	Detection of acetaminophen in meconium	ADHD	2.43 (95% CI, 1.41-4.21)
Baker 2020	Doubling of exposure	ADHD	1.10 (95% CI 1.02-1.19)
Chen 2019	Acetaminophen exposure in the second trimester	ADHD in offspring	1.19 (95% CI 1.00-1.40)
Chen 2019	Acetaminophen exposure in the first and second trimester	ADHD in offspring	1.28 (95% CI 1.00-1.64)
Chen 2019	Acetaminophen exposure in any trimester	ADHD in offspring	1.20 (95% CI 1.01-1.42)
Ji 2018	Below median maternal acetaminophen burden	ADHD diagnosis	1.58 (95% CI 1.02-2.46)
Ji 2018	Above median maternal acetaminophen burden	ADHD diagnosis	1.88 (95% CI 1.18, 3.00)
Gustavson 2021	Long-term exposure (29 days or more) to acetaminophen during pregnancy	ADHD diagnosis	2.02 (95% CI = 1.17–3.25)
Liew et al.,2014a	Maternal APAP use during pregnancy	HKD diagnosis, use of ADHD medications, or having ADHD-like behaviors at age 7 years	hazard ratio = 1.37; (95% CI, 1.19-1.59), hazard ratio = 1.29; (95% CI, 1.15-1.44), risk ratio = 1.13; (95% CI, 1.01-1.27)
Liew et al.,2016b	First-trimester APAP use	Poorer attention scores in childhood (-0.34, -0.63, -0.05 for overall attention; -0.25, -0.50, -0.01 for selective attention)	Not reported
Liew et al.,2016b	Prenatal exposure to APAP	Subnormal overall attention, selective attention difficulties, or parent-rated subnormal executive function (metacognition index)	OR 1.5, 95% CI 1.0-2.5; OR 1.5, 95% CI 1.0-2.4; OR 1.5, 95% CI 0.9-2.3
Liew et al.,2019	APAP use at the time of pregnancy	Childhood ADHD	OR = 1.34, 95% CI: 1.05-1.72
Thompson et al.,2014	APAP use during pregnancy	Higher total scores on all 3 SDQ formats	Not reported
Ystrom et al.,2017	Maternal APAP use for all indications, for more than 29 days	ADHD diagnosis	HR of 2.20 (95% CI 1.50-3.24)
Ystrom et al.,2017	APAP use for fever and infections, for 22 to 28 days	ADHD diagnosis	HR = 6.15 (95% CI 1.71-22.05)

Anand 2021:

- Cord blood acetaminophen >50th percentile was associated with higher odds of ADHD diagnosis (aOR: 2.10, 95% CI 1.43, 3.11).

Baker 2020:

- Detection of acetaminophen in meconium was associated with increased odds of ADHD (OR = 2.43, 95% CI, 1.41-4.21) and negative brain connectivity.
- A dose-response association was detected; each doubling of exposure increased the odds of ADHD by 10% (OR = 1.10, 95% CI 1.02-1.19).

Chen 2019:

- Acetaminophen exposure in the second trimester (OR = 1.19, 95% CI 1.00-1.40), first and second trimester (OR = 1.28, 95% CI 1.00-1.64), or any trimester (OR = 1.20, 95% CI 1.01-1.42) was associated with an increased risk of ADHD in offspring.

Ji 2018:

- Below median and above median levels of maternal acetaminophen burden were associated with a 58% and 88% increase in the odds of ADHD diagnosis, respectively (Model 6: OR for below median = 1.58, 95% CI 1.02-2.46; OR for above median 1.88, 95% CI 1.18, 3.00).

Gustavson 2021:

- Long-term exposure (29 days or more) to acetaminophen during pregnancy was associated with a two-fold increase in risk of ADHD diagnosis (aHR = 2.02, 95% CI = 1.17–3.25).
- In the sibling control model, the association between long-term acetaminophen use and ADHD in the child was no longer present (aHR = 1.06, 995% CI = 0.51–2.05).

Liew et al.,2014:

- Maternal acetaminophen use during pregnancy was associated with a higher risk of children receiving a hospital diagnosis of HKD (hazard ratio = 1.37; 95% CI, 1.19-1.59), use of ADHD medications (hazard ratio = 1.29; 95% CI, 1.15-1.44), or having ADHD-like behaviors at age 7 years (risk ratio = 1.13; 95% CI, 1.01-1.27).
- The associations were stronger with use in multiple trimesters and showed an exposure-response trend with increasing frequency of acetaminophen use during gestation.

Liew et al.,2016b:

- First-trimester APAP use was associated with poorer attention scores in childhood (mean difference -0.34, 95% CI -0.63, -0.05 for overall attention; -0.25, 95% CI -0.50, 0.01 for selective attention).
- Prenatal exposure to APAPl increased the risk of subnormal overall attention (OR 1.5, 95% CI 1.0-2.5), selective attention difficulties (OR 1.5, 95% CI 1.0-2.4), and parent-rated subnormal executive function (metacognition index, OR 1.5, 95% CI 0.9-2.3).
- Longer duration of APAP use in pregnancy further elevated the risks for subnormal overall attention or executive function.

Liew et al.,2019:

- Only APAP use at the time of pregnancy was associated with childhood ADHD (OR = 1.34, 95% CI: 1.05-1.72), and the effect estimates for the two negative control exposure periods (about 4 years before and 4 years after the pregnancy) were null.

Thompson et al.,2014:

- There were significantly higher total scores on all 3 SDQ formats is APAP was used during pregnancy, but there were no significant differences associated with any of the other drugs.

Ystrom et al.,2017:

- Maternal APAP use for all indications, for more than 29 days had a higher HR of 2.20 (95% CI 1.50-3.24).
- APAP use for fever and infections, for 22 to 28 days showed a strong and significant association with ADHD (HR = 6.15; 95% CI 1.71-22.05).

C. APAP and Adverse Birth Outcomes

In the section I review studies that investigated whether APAP use during pregnancy was associated with children having impaired learning, cognitive, or social outcomes (i.e., symptoms found in children diagnosed with ASD or ADHD).

Bertoldi 2020 (~)

The study is an observational study that estimates associations of prenatal and early-life exposure to acetaminophen in early childhood with cognitive, motor, and language skills in two birth cohorts.[437]

Information used to determine exposure: The study used questionnaires to determine acetaminophen use during pregnancy (Project Viva and Pelotas) and infancy (Project Viva).

Outcome definition and determination: The outcomes examined were cognitive, motor, and language skills in early childhood. The American Project Viva cohort assessed cognition at approximately 3 years using the Peabody Picture Vocabulary Test and the Wide Range Achievement of Visual Motor Abilities (WRAVMA). The Brazilian 2015 Pelotas Birth Cohort assessed cognition at 2 years using the INTERGROWTH-21st Neurodevelopment Assessment.

Control group: The control group would be children who were not exposed to acetaminophen during pregnancy or infancy.

Study size: The study included 1217 mother-child pairs from the American Project Viva cohort enrolled between 1999-2002 and 3818 mother-child pairs from the Brazilian 2015 Pelotas Birth Cohort.

Confounding factors or biases and how or if they were controlled: The study used linear regression to estimate associations of acetaminophen use during pregnancy (Project Viva and Pelotas) and infancy (Project Viva) with children's cognitive scores adjusted for maternal age, pre-pregnancy body mass index,

[437] Bertoldi et al. Associations of acetaminophen use during pregnancy and the first year of life with neurodevelopment in early childhood. Paediatr Perinat Epidemiol. 2020 May;34(3):267-277. doi: 10.1111/ppe.12632. Epub 2020 Jan 22. PMID: 31965601; PMCID: PMC7192774.

education, parity, race/ethnicity, smoking and alcohol use during pregnancy, depression during pregnancy, antibiotic and ibuprofen use during pregnancy, household income, and child's sex.

Limitations: The authors reported differences in outcomes and exposures between the Pelotas and Viva cohorts, making it challenging to ensure consistency in the findings. There was also a lack of information regarding dose and timing of exposures, and potential under-reporting in self-reported questionnaires or interviews. Confounding by indication was a concern, but adjustments were made for related variables. The cognitive/motor function tests used may be influenced by parental education and cultural/family aspects not accounted for. Differences in baseline covariates between participants and those lost to follow-up were observed, but analyses using inverse probability weighting (IPW) yielded similar results, suggesting minimal bias from loss to follow-up.

Results: In Project Viva, exposure to APAP in both the 1st and 2nd trimester of pregnancy was associated with lower WRAVMA drawing scores (β −1.51, 95% CI −2.92, −0.10). In Pelotas, exposure to APAP in both the 1st and 2nd trimester of pregnancy was not associated with INTER-NDA motor scores (β 0.02; 95% CI −0.05, 0.09) and was associated with higher INTER-NDA total scores (β 0.08, 95% CI 0.01, 0.16). Other comparisons did not show significant associations.

The study concluded that inconsistencies and lack of specificity of the findings did not clarify the research question considering that there is still a large variability and uncertainty to define the risk or safety in the use of acetaminophen related to cognition in early childhood. More studies using better exposure assessment and better confounding variables are needed to clarify these associations.

Bornehag 2018

The study is an observational population-based pregnancy cohort study.[438]

Information used to determine exposure: The study used two measures of exposure: (1) maternally reported number of APAP tablets taken between conception and enrollment; (2) APAP urinary concentration at enrollment.

Outcome definition and determination: The outcome examined was language development at 30 months, assessed by a nurse's evaluation and a parental questionnaire, including the number of words the child used (<25, 25–50, and >50). The main study outcome was parental report of use of fewer than 50 words, termed language delay (LD).

Control group: The control group would be children without language delay.

Study size: The study included 754 women who enrolled in the Swedish Environmental Longitudinal, Mother and child, Asthma and allergy (SELMA) study in pregnancy week 8–13.

Confounding factors or biases and how or if they were controlled: The study adjusted for maternal weight, mother's education, smoking, and week of enrollment.

[438] Bornehag et al. Prenatal exposure to acetaminophen and children's language development at 30 months. Eur Psychiatry. 2018 Jun;51:98-103. doi: 10.1016/j.eurpsy.2017.10.007. Epub 2018 Jan 10. PMID: 29331486.

Limitations: Reported limitations by the authors included unmeasured confounding, but they indicated that previous research and adjustments for potential confounders suggest that confounding by indication is unlikely. Exposure misclassification was a concern, as both urinary biomarkers and self-reported measures may underestimate exposure towards the null. Additionally, environmental sources of APAP may contribute to exposure variability. Outcome misclassification was a concern, although the validity of the language development instrument used is supported by consistent results from another study. They also reported that the study had limited power due to the small number of participants and low prevalence of language delay.

Results: The adjusted odds ratio (OR) for LD among girls whose mothers reported >6 vs. 0 APAP tablets was 5.92 (95% confidence interval (CI) 1.10–31.94). The OR for LD in girls whose mothers' urinary APAP was in the highest compared to the lowest quartile was 10.34 (95% CI 1.37–77.86).

Overall, the study reported that both the number of APAP tablets and urinary APAP concentration were associated with greater LD in girls but not in boys. The study concluded that given the prevalence of prenatal APAP use and the importance of language development, these findings, if replicated, would suggest that pregnant women should limit their use of this analgesic during pregnancy.

Brandlistuen 2013

The study is a sibling-controlled cohort study.

Information used to determine exposure: The study used maternal self-reported use of paracetamol during pregnancy. This is a study limitation, because self-reported maternal APAP use during pregnancy may be subject to recall bias or measurement error.

Outcome definition and determination: The outcomes examined were cognitive, motor, and language skills in early childhood. The study assessed cognition at 3 years using the McCarthy Scales of Children's Abilities, behavior at 5 years using the Strengths and Difficulties Questionnaire, and gross motor development at 3 years using the Ages and Stages Questionnaire.

Control group: The control group would be children who were not exposed to paracetamol during pregnancy.

Study size: The study included 48,631 children from the Norwegian Mother and Child Cohort Study (MoBa) whose mothers returned the 3-year follow-up questionnaire by May 2011. Within this sample were 2919 same-sex sibling pairs who were used to adjust for familial and genetic factors. Among the 2919 pairs of siblings with complete data on paracetamol exposure: 134 (4.6%) were discordant for exposure >28 days; 805 (27.6%) were discordant for 1–27 days of exposure; and 1980 were concordant (1224 were both unexposed, 756 were both exposed).

Confounding factors or biases and how or if they were controlled: The study adjusted for a number of factors, including maternal age, education, smoking, alcohol use, caffeine intake, pre-pregnancy body mass index (BMI), parity, marital status, household income, life events during pregnancy, symptoms of depression/anxiety during pregnancy, use of other pain medication during pregnancy, fever/infection during pregnancy, child sex, gestational age at birth, birth weight, and breastfeeding duration.

Limitations: Reported limitations included the relatively low participation rate in the MoBa study and selection bias. Reliance on self-reporting for assessment could lead to underestimation due to social desirability and stigma. The study was unable to consider dosage and distinguish between continuous and sporadic APAP use during pregnancy due to limited data. There was also a possibility of residual confounding factors, such as unreported infections or illnesses, influencing the observed effects.

Results: The study reported that children exposed to prenatal APAP for more than 28 days had poorer gross motor development (β 0.24), communication (β 0.20), externalizing behavior (β 0.28), internalizing behavior (β 0.14), and higher activity levels (β 0.24) compared with unexposed children. Children exposed prenatally to short-term use of APAP (1–27 days) also had poorer gross motor outcomes (β 0.10), but the effects were smaller than with long-term use.

Overall, the study concluded that children exposed to long-term use of APAP during pregnancy may be at increased risk of neurodevelopmental disorders.

Golding 2019 (~)
The study is a longitudinal cohort study.[439]

Information used to determine exposure: The study used data collected by the Avon Longitudinal Study of Parents and Children (ALSPAC) at 32-weeks gestation and referring to the period from 18 to 32 weeks, which identified 43.9% of women having taken paracetamol.

Outcome definition and determination: The outcomes examined were neurocognitive outcomes in the child, including behavior, language, and cognitive development.

Control group: The control group would be children whose mothers did not take paracetamol during pregnancy.

Study size: The study included data from the ALSPAC cohort, which recruited over 14,000 pregnant women with expected delivery dates between April 1991 and December 1992.

Confounding factors or biases and how or if they were controlled: The study used an exposome analysis first to determine the background factors associated with pregnant women taking paracetamol, and then allowed for those factors to assess associations with child outcomes. The study identified 15 variables independently associated with taking paracetamol in this time period, which were used as potential confounders.

Limitations: Reported limitations included collection constraints, potentially leaving out other important confounding factors that could impact the conclusions. Additionally, the approach used to identify confounders was stringent, and relaxing the selection criteria may have revealed a wider range of confounding variables. The examination of APAP use was limited to the period between 18 and 32 weeks

[439] Golding et al. Associations between paracetamol (acetaminophen) intake between 18 and 32 weeks gestation and neurocognitive outcomes in the child: A longitudinal cohort study. Paediatr Perinat Epidemiol. 2020 May;34(3):257-266. doi: 10.1111/ppe.12582. Epub 2019 Sep 15. PMID: 31523834; PMCID: PMC7217049.

of pregnancy, excluding early and late stages. The available data on the frequency of APAP use were insufficient, particularly for analyzing frequent users. The analysis also focused on continuous data for the outcomes considered, potentially missing strong or dose effects that may be present in the tails of the distribution.

Results: Adjusted finding identified 12 independent associations with APAP use at $P < .05$, four of which were at $P < .0001$ (all related to child behaviors reported by the mother at 42 and 47 months, *e.g.*, conduct problems: adjusted mean score +0.22 (95% confidence interval 0.10-0.33). These findings included measures of hyperactivity, attention, conduct problems, and total behavioral difficulties, mostly in the pre-school period.

Overall, the study reports that of 135 neurocognitive variables considered, adjusting for the likelihood of false discovery, the study identified 56 outcomes for adjusted analyses. The study concluded that if paracetamol use in mid-to-late pregnancy has an adverse effect on child neurocognitive outcome, it appears to mainly relate to the pre-school period.

Laue 2019 (~)
The study is a prospective birth cohort study.[440]

Information used to determine exposure: The study used concentrations of acetaminophen measured in meconium, which more objectively captures exposure of the fetus than maternal report.

Outcome definition and determination: The outcomes examined were neurocognitive development in early childhood, including cognitive, motor, and language skills. At age 6-8 years, children completed a set of subtests from the Wechsler Intelligence Scale for Children, 4th edition.

Control group: The control group would be children who were not exposed to acetaminophen during pregnancy.

Study size: The study included data from the GESTE cohort, which recruited pregnant women in Canada. Women were recruited between 2007 and 2009 during the first trimester of pregnancy and at delivery (n = 800). At age 6–8 years, 363 children completed a series of neurocognitive tests at the study site, 195 of whom had stored meconium. After excluding individuals with missing covariate data, the final sample size was 118 children.

Confounding factors or biases and how or if they were controlled: The study adjusted for a number of factors, including maternal age, education, smoking, alcohol use, pre-pregnancy body mass index (BMI), parity, marital status, household income, life events during pregnancy, symptoms of depression/anxiety during pregnancy, use of other pain medication during pregnancy, fever/infection during pregnancy, child sex, gestational age at birth, birth weight, and breastfeeding duration.

Limitations: The study was small, having only 118 children. Based on exposed (n=63) versus unexposed (n = 55), the study size is currently only powered to measure a ~7-fold increase in risk between two groups.

[440]Laue et al. Association Between Meconium Acetaminophen and Childhood Neurocognitive Development in GESTE, a Canadian Cohort Study. Toxicol Sci. 2019 Jan 1;167(1):138-144. doi: 10.1093/toxsci/kfy222. PMID: 30202886; PMCID: PMC6317422.

To detect a two-fold increase in risk, n = 1608, assuming 1 in 36 background incidence, a = 0.05 and power = 80%.

Results: In those exposed, with APAP in the meconium, the median concentration was 59.9 ng/g. Exposure was categorized into 3 levels: below the analytical limit of detection (≤ LOD, n = 55), low (> LOD, ≤ 59.9 ng/g, n = 32), and high (> 59.9 ng/g, n = 31). The effect of *in utero* APAP exposure on the Coding subtest was marginally significantly different among boys and girls, with girls performing significantly better on the task with higher levels of acetaminophen compared with girls with undetectable levels of exposure (βgirls, low = 2.83 [0.97, 4.70], βgirls, high = 1.95 [-0.03, 3.93], βboys, low = .02 [-1.78, 1.81], βboys, high = -.39 [-2.09, 1.31], interaction = .06). Effect modification by child sex was not observed on other subtests. The study concluded that these results do not support prior reports of adverse neurodevelopmental effects of *in utero* exposure to acetaminophen.

Overall, in fully adjusted models, *in utero* exposure to acetaminophen was not statistically significantly associated with decreased scores on any of the examined subtests in all children combined (n = 118).

Liew 2016a
The study is a prospective cohort study.[441]

Information used to determine exposure: The study used maternal self-reported acetaminophen use during pregnancy, which was assessed prospectively via 3 telephone interviews. This is a study limitation, because self-reported maternal APAP use during pregnancy may be subject to recall bias or measurement error.

Outcome definition and determination: The outcome examined was child IQ at age 5. Child IQ was assessed using the Wechsler Primary and Preschool Scales of Intelligence-Revised (WPPSI-R) administered by trained psychologists.

Control group: The control group would be children whose mothers did not use acetaminophen during pregnancy.

Study size: The study included data from 1,491 mothers and children enrolled in the Danish National Birth Cohort (DNBC; 1996-2002).

Confounding factors or biases and how or if they were controlled: The study employed linear regression analysis, adjusting for maternal IQ and other confounding factors, and assessed interactions between acetaminophen and indications for use.

Limitations: There was the potential for exposure misclassification, residual confounding, and lack of generalizability to less healthy children or different populations.

Results: The study reported that both maternal fever in pregnancy and acetaminophen use were associated with child IQ. Children born to mothers using acetaminophen without reporting fever scored on average 3.4 points lower on performance IQ compared with offspring of mothers who neither experienced fever

[441] Liew et al. Prenatal Use of Acetaminophen and Child IQ: A Danish Cohort Study. Epidemiology. 2016 Nov;27(6):912-8. doi: 10.1097/EDE.0000000000000540. PMID: 27479646.

nor took acetaminophen. Exposures for more than one trimester impacts IQ (mean difference = -3.1, 95% CI -5.7 to -0.45). Use from 1-5 week are impacts IQ (mean difference = -3.1, 95% CI -5.6 to -0.68)

The study concluded that maternal acetaminophen use during pregnancy was associated with lower performance IQ in 5-year-olds.

Parker 2019 (~)
The study is a longitudinal study.[442]

Information used to determine exposure: The study used maternal self-reported acetaminophen use during pregnancy, which was assessed via a standardized maternal interview completed 1 year after delivery on average. This is a study limitation, because self-reported maternal APAP use during pregnancy may be subject to recall bias or measurement error.

Outcome definition and determination: The outcomes examined were childhood behavior problems at ages 6-12 years. Measures of childhood behavior were obtained via mother- and teacher-report, using the Child Behaviour Checklist and Teacher Report Form.

Control group: The control group would be children whose mothers did not use acetaminophen during pregnancy.

Study size: The study included data from 560 mother-child pairs with data on illnesses and medication use during pregnancy and neurodevelopmental assessments during childhood.

Confounding factors or biases and how or if they were controlled: The study employed linear and log-binomial models to calculate adjusted mean differences (MD) and risk ratios (RR), respectively, for internalizing, externalizing, and total behavior problems comparing acetaminophen users to non-users. Stabilized inverse probability weights were used to account for loss to follow-up, and adjustments for indication were made.

Limitations: The limitations of the study include retrospective self-reported measures of APAP exposure, a focus on the first 5 months of pregnancy, potential inaccuracies in medication reporting with longer interview intervals, possible dependent misclassification, and the lack of data on important confounders such as maternal stress, IQ, and behavioral problems.

Results: Approximately 60% (n = 354) of women reported use of APAP during pregnancy. Acetaminophen use during pregnancy was associated with increased total behavior problem scores and a higher risk of clinical behavior problems according to mother reports (MD 2.2, 95% CI 0.3, 4.1 and RR 1.93, 95% CI 0.99, 3.76), but not according to teacher reports. Weighting for participation and adjusting for indications of APAP use had minimal impact on the associations, with attenuated effects on mother-reported behavior problem scores and risk of clinical behavior problems (MD 0.1, 95% CI −2.1, 2.3 and RR 1.31, 95% CI 0.67, 2.58).

[442] Parker et al. Maternal acetaminophen use during pregnancy and childhood behavioural problems: Discrepancies between mother- and teacher-reported outcomes. Paediatr Perinat Epidemiol. 2020 May;34(3):299-308. doi: 10.1111/ppe.12601. Epub 2019 Nov 6. PMID: 31693212; PMCID: PMC7192789.

The study concluded that APAP use during pregnancy was weakly associated with mother-reported behavior problems and not associated with teacher-reported problems.

Rifas-Shiman 2019 (~)
The study is a longitudinal study.[443]

Information used to determine exposure: The study used maternal self-reported acetaminophen and ibuprofen use during pregnancy and infancy, which was assessed via questionnaires. This is a study limitation, because self-reported maternal APAP use during pregnancy may be subject to recall bias or measurement error.

Outcome definition and determination: The outcomes examined were childhood executive function and behavior problems at ages 6-12 years. Measures of childhood behavior were obtained via parent- and teacher-report, using the Behavior Rating Inventory of Executive Function (BRIEF) and the Strengths and Difficulties Questionnaire (SDQ), with higher scores indicating worse functioning for both.

Control group: The control group would be children whose mothers did not use acetaminophen or ibuprofen during pregnancy or infancy.

Study size: The study included data from 1,225 mother-child pairs from Project Viva, a pre-birth cohort study.

Confounding factors or biases and how or if they were controlled: The study examined associations of acetaminophen and ibuprofen use during pregnancy and infancy with mid-childhood neurobehavioral outcomes using linear regression models adjusted for potential confounders.

Limitations: Reported limitations included imprecise dose information, limited assessment of analgesic intake during specific pregnancy periods, lack of data on analgesic use during lactation, potential residual confounding by indication, and differences in baseline covariates between participants and those lost to follow-up.

Results: the study reports that during pregnancy, 46.1% of mothers used acetaminophen ≥10 times and 18.4% used any ibuprofen. In the first year, 65.3% and 39.6% of infants received acetaminophen and ibuprofen ≥6 times, respectively. Higher (≥10 vs <10 times) prenatal acetaminophen (β 1.64 points; 95% confidence interval [CI] 0.59, 2.68) and any ibuprofen (β 1.56, 95% CI 0.19, 2.92) were associated with higher parent-rated BRIEF global scores. Infancy exposure (≥6 vs <6 times) to acetaminophen (β 1.69, 95% CI 0.51, 2.87) and ibuprofen (β 1.40, 95% CI 0.25, 2.55) were associated with higher parent-rated BRIEF GEC scores but associations with teacher-rated scores were weaker.

The study concluded that prenatal and early-life exposure to APAP and ibuprofen were associated with poorer executive function and behavior in childhood. These findings highlight the need for further research on the mechanisms through which analgesics may act on fetal and child brain development.

[443] Rifas-Shimanet al. Associations of prenatal or infant exposure to acetaminophen or ibuprofen with mid-childhood executive function and behaviour. Paediatr Perinat Epidemiol. 2020 May;34(3):287-298. doi: 10.1111/ppe.12596. Epub 2019 Oct 21. PMID: 31637744; PMCID: PMC7170759.

Stergiakouli 2016
The study was a prospective birth cohort study.[444]

Information used to determine exposure: Acetaminophen use was assessed by questionnaire completion at 18 and 32 weeks of pregnancy and when the child was 61 months old.

Outcome definition and determination: The main outcome was maternal reports of behavioral problems using the Strengths and Difficulties Questionnaire (SDQ) when the children were 7 years old.

Control group: The control group consisted of children whose mothers did not report acetaminophen use during pregnancy.

Study size: The study included 7,796 mothers enrolled in the Avon Longitudinal Study of Parents and Children (ALSPAC) between 1991 and 1992 along with their children and partners.

Confounding factors or biases and how or if they were controlled: The study adjusted for maternal postnatal or partner's acetaminophen use in the analysis.

Limitations: Limitations of the study include the lack of information on indications, dosage, and duration of APAP use, absence of genetic data from partners, limited explanatory power of polygenic risk scores, reliance on maternal reports introducing potential bias, and delayed collection of postnatal acetaminophen use data.

Results: Maternal prenatal acetaminophen use at 18 and 32 weeks of pregnancy was associated with increased odds of conduct problems, hyperactivity symptoms, emotional symptoms, and total difficulties in offspring. The risk ratios (RR) for conduct problems and hyperactivity symptoms were 1.42 (95% CI, 1.25-1.62) and 1.31 (95% CI, 1.16-1.49), respectively. APAP use at 32 weeks was also associated with higher odds of emotional symptoms (RR, 1.29; 95% CI, 1.09-1.53) and total difficulties (RR, 1.46; 95% CI, 1.21-1.77). There was no association found with maternal postnatal or paternal/partner APAP use.

The study concluded that children exposed to acetaminophen prenatally are at increased risk of multiple behavioral difficulties.

Tovo-Rodrigues 2020
The study was a prospective longitudinal study that used data from the 2004 Pelotas Birth Cohort, Brazil.[445]

[444] Stergiakouli et al. Association of Acetaminophen Use During Pregnancy With Behavioral Problems in Childhood: Evidence Against Confounding. JAMA Pediatr. 2016 Oct 1;170(10):964-970. doi: 10.1001/jamapediatrics.2016.1775. PMID: 27533796; PMCID: PMC5300094.
[445] Tovo-Rodrigues et al. Low neurodevelopmental performance and behavioural/emotional problems at 24 and 48 months in Brazilian children exposed to acetaminophen during foetal development. Paediatr Perinat Epidemiol. 2020 May;34(3):278-286. doi: 10.1111/ppe.12649. Epub 2020 Mar 20. PMID: 32196712.

Information used to determine exposure: Maternal use of medication during pregnancy was retrospectively assessed using a standardized questionnaire applied at the perinatal evaluation. Acetaminophen use was defined as at least once during pregnancy, regardless of the dose used.

Outcome definition and determination: The outcomes were low neurodevelopmental performance at 24 months and emotional/behavioral problems at 48 months of life in children from the 2004 Pelotas Birth Cohort (Brazil). Neurodevelopment was evaluated at 24 months using Battelle's Developmental Inventory (BDI) and behavioral/emotional problems were assessed at 48 months using the Child Behaviour Checklist (CBCL).

Control group: The control group consisted of children whose mothers did not report acetaminophen use during pregnancy.

Study size: At 24 months, a total of 3,727 children had valid information for acetaminophen exposure and BDI, and at 48 months, the number of children with valid information for exposure and CBCL was 3,624.

Confounding factors or biases and how or if they were controlled: The study adjusted for several family and maternal sociodemographic and health factors, medication use during pregnancy, and the sex of the child in their analysis.

Limitations: Reported limitations included retrospective data collection may have led to recall difficulties and underreporting of acetaminophen use during pregnancy; the study may have lacked sufficient power; confounding by indication and residual confounding are possible; postnatal acetaminophen exposure was not assessed.

Results: Prenatal acetaminophen exposure did not show any association with neurodevelopmental performance at 24 months or emotional and behavioral problems at 48 months, as assessed by the BDI and CBCL measures, respectively, in the adjusted models.

The authors conclude that they cannot confirm the existence of an association between acetaminophen used during pregnancy and low neurodevelopmental performance at 24 months and emotional/behavioral problems at 48 months of life based on their results.

Tronnes 2019

The study is a prospective cohort study using data from the Norwegian Mother and Child Cohort Study.[446]

Information used to determine exposure: The exposure was determined by self-reported maternal use of paracetamol during pregnancy, as reported in questionnaires. This is a study limitation, because self-reported maternal APAP use during pregnancy may be subject to recall bias or measurement error.

Outcome definition and determination: The outcomes were parent-reported communication skills, behavior, and temperament in preschool-aged children, assessed using standardized questionnaires.

[446] Trønnes et al. Prenatal paracetamol exposure and neurodevelopmental outcomes in preschool-aged children. Paediatr Perinat Epidemiol. 2020 May;34(3):247-256. doi: 10.1111/ppe.12568. Epub 2019 Aug 25. PMID: 31448449; PMCID: PMC8285062.

Control group: The control group consisted of children whose mothers did not report paracetamol use during pregnancy.

Study size: The study included 32,934 children.

Confounding factors or biases and how or if they were controlled: The study used inverse probability weights and robust standard errors to control for potential confounders such as maternal age, education, marital status, pre-pregnancy BMI, smoking habits, alcohol use, symptoms of anxiety and depression, maternal health conditions during pregnancy, and concomitant medication use.

Limitations: Study limitations include low participation rates with potential self-selection bias, reliance on parent-reported data introducing misclassification, and lack of information on formulation and dose of acetaminophen.
Results: In our study of 32,934 children, prenatal exposure to paracetamol varied across trimesters: 25.4% in one trimester, 15.1% in two trimesters, and 5.4% in all three trimesters. Exposure in two trimesters was associated with lower shyness scores (β −0.62, 95% CI −1.05 to −0.19), while exposure in three trimesters increased the risk of internalizing behavior problems (RR 1.36, 95% CI ,1.02-1.80) and borderline externalizing behavior problems (RR 1.22, 95% CI, 0.93-1.60). Exposure in the second and third trimesters was also linked to lower shyness scores (β −0.32, 95% CI −0.66 to 0.02). Sensitivity analyses highlighted the potential influence of unmeasured confounders biasing the effect estimates.

The authors conclude that an association was found between APAP use in multiple trimesters and lower shyness and greater internalizing behavior in preschool-aged children. They also suggest that chance or unmeasured confounding cannot be ruled out as possible explanations for these findings.

Vlenterie 2016
The study was a prospective cohort study using data from the Norwegian Mother and Child Cohort Study.[447]

Information used to determine exposure: Paracetamol use was classified into short-term (< 28 days) and long-term (≥ 28 days) of exposure.

Outcome definition and determination: The outcomes were communication problems and delayed motor milestone attainment among children at 18 months of age.

Control group: The control group consisted of children whose mothers did not report paracetamol use during pregnancy.

Study size: Of the 51,200 pregnancies included in the study, 40.5% of mothers (n = 20,749) used paracetamol at least once during pregnancy.

[447] Vlenterie et al. Neurodevelopmental problems at 18 months among children exposed to paracetamol in utero: a propensity score matched cohort study. Int J Epidemiol. 2016 Dec 1;45(6):1998-2008. doi: 10.1093/ije/dyw192. PMID: 27585674; PMCID: PMC5841617.

Confounding factors or biases and how or if they were controlled: The study applied propensity score (PS) matching to examine associations between prenatal paracetamol exposure and neurodevelopmental problems among children at 18 months of age.

Limitations: Reported limitations included the possibility of selection bias in the study cohort, limited generalizability to women with severe forms of headaches or migraines, inability to include information on severity and type of infections during pregnancy, potential residual confounding by unmeasured variables, increased risk of chance findings due to exploring multiple outcomes, reliance on parent-reported behavior outcomes prone to misclassification, and substantial loss to follow-up at the 18-month questionnaire introducing potential selection bias.

Results: The study reported that in the PS-matched analyses, long-term paracetamol exposure during pregnancy was associated with communication problems [odds ratio (OR): 1.38, 95% CI 0.98–1.95] and delayed motor milestone attainment (OR: 1.35, 95% CI 1.07–1.70). The study did not observe increased risks after short-term exposure. Sensitivity analyses for several indications showed similar effects as the PS-matched analyses, suggesting no confounding by indication.

The study concluded that long-term exposure to paracetamol in utero was associated with modestly increased risks of motor milestone delay and impaired communication skills among children at 18 months.

Weight of the Evidence of the Association of APAP with Other Cognitive or Social Outcomes

Overall, the studies above constitute **some evidence that APAP use in pregnancy causes developmental toxicity.** This is supported by reports of impaired learning, cognitive, or social outcomes in offspring exposed to APAP during pregnancy.

Based on a weight-of-evidence analysis of the studies included in the report, **the evidence for an association between prenatal APAP exposure and adverse neurodevelopmental outcomes in children can be considered moderate.** Several studies have reported significant associations between prenatal APAP exposure and poorer cognitive, motor, language, and behavioral outcomes in children. However, the evidence is not consistent across all studies, and some studies did not find significant associations or found mixed results.

APAP has a moderate association with an increased risk of adverse neurodevelopmental outcomes in children. The data from these above studies are summarized below:

Study	Exposure	Outcome	Measure	Result
Bertoldi 2020	APAP use in both the 1st and 2nd trimester of pregnancy	WRAVMA drawing scores in Project Viva	Beta coefficient	-1.51 (95% CI -2.92, -0.10)
Bornehag 2018	Number of APAP tablets and urinary APAP concentration	Language delay in girls	Odds ratio	5.92 (95% CI 1.10–31.94) and 10.34 (95% CI 1.37–77.86)

Brandlistuen 2013	Prenatal APAP use for more than 28 days	Gross motor development, communication, externalizing behavior, internalizing behavior, and higher activity levels	Beta coefficient	0.24, 0.20, 0.28, 0.14, and 0.24 respectively
Golding 2019	APAP use at P < .05	Child behaviors reported by the mother at 42 and 47 months	Adjusted mean score	+0.22 (95% CI 0.10-0.33) for conduct problems
Laue 2019	In utero APAP exposure on the Coding subtest	Coding subtest scores among boys and girls	Beta coefficient	-0.39 (95% CI -2.09, 1.31) for boys and 1.95 (95% CI -0.03, 3.93) for girls
Liew 2016a	Maternal APAP use without reporting fever	Performance IQ	Mean difference	-3.1 (95% CI -5.7 to -0.45)
Parker 2019	Prenatal APAP exposure	Total behavior problem scores and clinical behavior problems according to mother reports	Mean difference and risk ratio	2.2 points (95% CI ,0.3-4.1) and 1.93 (95% CI ,0.99-3.76) respectively
Rifas-Shiman 2019	Higher prenatal APAP and any ibuprofen	Parent-rated BRIEF global scores	Beta coefficient	1.64 points (95% CI ,0.59-2.68) and 1.56 points (,95% CI ,0.19-2.92) respectively
Stergiakouli 2016	Maternal prenatal APAP use at 18 and 32 weeks of pregnancy	Conduct problems, hyperactivity symptoms, emotional symptoms, and total difficulties in offspring	Risk ratio	at 18 weeks: 1.42 (95% CI, 1.25-1.62) and 1.31 (95% CI, 1.16-1.49), at 32 weeks: RR 1.29 (95% CI, 1.09-1.53) and RR 1.46; (95% CI, 1.21-1.77).
Tovo-Rodrigues2020	Prenatal acetaminophen exposure	Neurodevelopmental performance at 24 months or emotional and behavioral problems at 48months	None	
Tronnes 2019	Exposure in three trimesters	Internalizing behavior problems and borderline externalizing behavior problems	Risk ratio	RR 1.36 (95% CI ,1.02-1.80) and RR 1.22 (95% CI, 0.93-1.60).
Vlenterie 2016	Long-term paracetamol exposure during pregnancy	Communication problems and delayed motormilestone attainment	Odds ratio	OR: 1.35 (95% CI 1.07–1.70).

Bertoldi 2020: Exposure to APAP in both the 1st and 2nd trimester of pregnancy was associated with lower WRAVMA drawing scores (β −1.51, 95% CI −2.92, −0.10) in Project Viva.

Bornehag 2018: The adjusted odds ratio (OR) for LD among girls whose mothers reported >6 vs. 0 APAP tablets was 5.92 (95% confidence interval (CI) 1.10–31.94). The OR for LD in girls whose mothers' urinary APAP was in the highest compared to the lowest quartile was 10.34 (95% CI 1.37–77.86).

Brandlistuen 2013: Children exposed to prenatal APAP for more than 28 days had poorer gross motor development (β 0.24), communication (β 0.20), externalizing behavior (β 0.28), internalizing behavior (β 0.14), and higher activity levels (β 0.24) compared with unexposed children.

Golding 2019: Adjusted finding identified 12 independent associations with APAP use at $P < .05$, four of which were at $P < .0001$ (all related to child behaviors reported by the mother at 42 and 47 months, e.g., conduct problems: adjusted mean score +0.22 (95% confidence interval 0.10-0.33).

Laue 2019: The effect of in utero APAP exposure on the Coding subtest was marginally significantly different among boys and girls, with girls performing significantly better on the task with higher levels of acetaminophen compared with girls with undetectable levels of exposure (βgirls, low = 2.83 [0.97, 4.70], βgirls, high = 1.95 [-0.03, 3.93], βboys, low = .02 [-1.78, 1.81], βboys, high = -.39 [-2.09, 1.31], pinteraction = .06).

Liew 2016a: Children born to mothers using acetaminophen without reporting fever scored on average 3.4 points lower on performance IQ compared with offspring of mothers who neither experienced fever nor took acetaminophen (mean difference = -3.1, 95% CI -5.7 to -0.45).

Parker 2019: Acetaminophen use during pregnancy was associated with increased total behavior problem scores and a higher risk of clinical behavior problems according to mother reports (mean difference = 2.2 points; 95% CI ,0.3-4.1 and risk ratio = 1.93, 95% CI ,0.99-3.76).

Rifas-Shiman 2019: Higher prenatal acetaminophen and any ibuprofen were associated with higher parent-rated BRIEF global scores (β = 1.64 points; 95% CI ,0.59-2.68 and β = 1.56 points; ,95% CI ,0.19-2.92).

Stergiakouli 2016: Maternal prenatal acetaminophen use at 18 and 32 weeks of pregnancy was associated with increased odds of conduct problems, hyperactivity symptoms, emotional symptoms, and total difficulties in offspring (risk ratio = 1.42, ,95% CI ,1.25-1.62; risk ratio = ,1.,31; ,95% CI ,1.,16-1.,49; risk ratio = ,1.,29; ,95% CI ,1.,09-1.,53; and risk ratio = ,1.,46; ,95% CI ,1.,21-1.,77 respectively)

Tovo-Rodrigues 2020: This study did not find any association between prenatal acetaminophen exposure and neurodevelopmental performance at 24 months or emotional and behavioral problems at 48 months in Brazilian children.

Tronnes 2019: This study found that exposure to acetaminophen in three trimesters increased the risk of internalizing behavior problems (RR 1.36, 95% CI 1.02-1.80) and borderline externalizing behavior

problems (RR 1.22, 95% CI 0.93-1.60) in preschool-aged children from the Norwegian Mother and Child Cohort Study.

Vlenterie 2016: This study found that long-term paracetamol exposure during pregnancy was associated with communication problems (OR 1.38, 95% CI 0.98-1.95) and delayed motor milestone attainment (OR 1.35, 95% CI 1.07-1.70) among children at 18 months of age from the Danish National Birth Cohort.

D. Summary of AOP and APAP Causation of Neurodevelopmental Disorders (Meta-Analysis)

There are several meta-analyses that have been performed on the available epidemiological studies. These studies draw upon the above studies and meta-analysis and also report increased risks for ASD and ADHD with perinatal exposures to APAP.

Alemany 2021
This meta-analysis aimed to assess the association between maternal use of acetaminophen during pregnancy and the risk of attention deficit/hyperactivity disorder (ADHD) and autism spectrum disorder (ASD) in offspring.[448] The study included 6 European cohort studies with a total of 73,881 mother-child pairs.

The results showed that maternal use of acetaminophen during pregnancy was associated with an increased risk of ADHD in offspring (pooled relative risk [RR] = 1.29, 95% confidence interval [CI] = 1.20–1.39). The association was stronger for male offspring (RR = 1.35, 95% CI = 1.23–1.48) than for female offspring (RR = 1.18, 95% CI = 1.06–1.32). Maternal use of acetaminophen during pregnancy was also associated with an increased risk of ASD in offspring (pooled RR = 1.21, 95% CI = 1.08–1.36).

The conclusion of the meta-analysis is that maternal use of acetaminophen during pregnancy is associated with an increased risk of ADHD and ASD in offspring.

Gou 2019
This study examined association between maternal APAP use during pregnancy and the risk of ADHD in offspring via meta-analysis.[449] Eight cohort studies with a total of 244,940 participants were included for this analysis.

Maternal exposure to APAP during pregnancy increased the risk of ADHD in offspring with a pooled adjusted risk ratio of 1.25 (95% confidence interval = 1.17-1.34). Children exposed prenatally to acetaminophen in the third trimester seemed to have the greatest risk of developing ADHD (risk ratio: 1.26; 95% confidence interval = 1.08-1.47). In addition, a longer duration of maternal acetaminophen use during pregnancy was correlated with a higher risk ratio.

This study supports that increased use of APAP during pregnancy is associated with a higher risk of ADHD development in children due to fetal exposure. Children whose mothers used acetaminophen for

[448] Alemany et al. Prenatal and postnatal exposure to acetaminophen in relation to autism spectrum and attention-deficit and hyperactivity symptoms in childhood: Meta-analysis in six European population-based cohorts. Eur J Epidemiol. 2021 Oct;36(10):993-1004. doi: 10.1007/s10654-021-00754-4. Epub 2021 May 28. PMID: 34046850; PMCID: PMC8542535.
[449] Gou et al. Association of maternal prenatal acetaminophen use with the risk of attention deficit/hyperactivity disorder in offspring: A meta-analysis. Aust N Z J Psychiatry. 2019 Mar;53(3):195-206. doi: 10.1177/0004867418823276. Epub 2019 Jan 17. PMID: 30654621.

28 or more days during gestation had a higher risk of developing ADHD (risk ratio: 1.63; 95% confidence interval = 1.23-2.16). One limitation noted was that these findings are based on three studies (Liew et al., 2014; Stergiakouli et al., 2016; Ystrom et al., 2017).

The study also reported that the risk of ADHD was higher when APAP was used during the third trimester compared to the first and second trimesters. This could be related to the rapid growth and structural changes happening in the fetal brain during that time.

Gender differences were also explored, and some studies suggested stronger effects in girls. The prevalence of ADHD is generally higher in boys than in girls, but the reasons for this difference are not fully understood and may involve biological factors and susceptibility to early-life stressors. The study reports conflicting findings on gender differences in ADHD, calling for further research to better understand the relationship.

The meta-analysis concludes that there is an association between maternal APAP use during pregnancy and an increased risk of ADHD in offspring. The timing and duration of APAP use during pregnancy has a major effect on the risk of ADHD.

Kim 2019

This study is an umbrella review of meta-analyses that examined the associations between environmental risk factors, biomarkers, and autism spectrum disorder.[450] The authors used various statistical tests to assess the strength and validity of the evidence for each association. The eligible meta-analyses were classified into five levels of evidence based on a series of statistical tests and criteria. The levels of evidence were: convincing (class I), highly suggestive (class II), suggestive (class III), weak (class IV), and not significant. The criteria included the p value, the number of cases, the heterogeneity, the small study effects, the excess significance bias, the credibility ceiling, and the prediction interval. The authors also did sensitivity analyses to check the robustness of the associations. They found that only seven environmental risk factors, mostly related to maternal age, metabolic syndrome, and antidepressant use, had convincing evidence of association with autism spectrum disorder.

The authors reported that APAP use during pregnancy was one of the environmental risk factors that was graded as highly suggestive evidence (class II) of association with autism spectrum disorder. The random effects summary estimate for this association was a relative risk of 1.20 (95% CI 1.14–1.26) based on five cohort studies with more than 1000 cases of autism spectrum disorder. However, this association showed signs of small study effects and was not significant under 10% credibility ceiling, meaning that it could be affected by bias or confounding factors.

Kim 2020

The article is an umbrella review of meta-analyses that examines the association between potential environmental risk factors, environmental protective factors, or peripheral biomarkers with ADHD. [451] The eligible meta-analyses were classified according to the strength of the evidence of potential environmental risk factors, environmental protective factors, and peripheral biomarkers for ADHD into

[450] Kim et al. Environmental risk factors and biomarkers for autism spectrum disorder: an umbrella review of the evidence. Lancet Psychiatry. 2019 Jul;6(7):590-600. doi: 10.1016/S2215-0366(19)30181-6. PMID: 31230684.

[451] Kim et al. Environmental risk factors, protective factors, and peripheral biomarkers for ADHD: an umbrella review. Lancet Psychiatry. 2020 Nov;7(11):955-970. doi: 10.1016/S2215-0366(20)30312-6. PMID: 33069318.

five classes: convincing (class I), highly suggestive (class II), suggestive (class III), weak (class IV), and not significant (NS). Criteria for each level of evidence were p values under a random effects model, the number of ADHD cases, the statistical significance of the largest study, the I^2 statistic, small study effects, excess significance bias, random effects summary estimate under a 10% credibility ceiling, and the 95% prediction interval.

The study found that maternal pre-pregnancy obesity, childhood eczema, hypertensive disorders during pregnancy, pre-eclampsia, and maternal APAP exposure during pregnancy were strongly associated with ADHD. Maternal APAP exposure during pregnancy was found to have a "convincing" association with ADHD. The study found that evidence of association was convincing (class I) for maternal APAP exposure during pregnancy (RR 1.25, 95% CI 1.17 to 1.34). The authors note that there is a possibility of residual confounding, as not all potential confounders were adjusted for in the included studies.

Patel 2022

The article is a systematic review of the long-term safety of prenatal and neonatal exposure to paracetamol, a common antipyretic and analgesic drug.[452] The authors searched three electronic databases for original research studies that examined the occurrence of neurodevelopmental, atopic or reproductive adverse outcomes in children exposed to APAP in the womb or within the first four weeks of life.They included 30 studies in the final review, most of which were observational epidemiologic and cohort studies. The majority of the studies focused on prenatal exposure, while only three studies reported on neonatal exposure.

The authors found that prenatal APAP exposure was associated with an increased risk of neurodevelopmental, atopic and reproductive adverse outcomes in children, such as asthma, wheezing, eczema, behavior problems, attention deficit hyperactivity disorder (ADHD), autism spectrum disorder (ASD), reduced anogenital distance (AGD) and delayed pubertal development. They also found that neonatal paracetamol exposure was possibly correlated with long-term adverse outcomes in one study.

The authors suggest caution in the use of APAP during pregnancy and early life.

Ricci 2023

This systematic review and meta-analysis reviewed 22 studies including 23 cohorts with a total of 367,775 participants that aimed to summarize the literature on the association between in utero acetaminophen exposure and child neurodevelopmental outcomes and assess the extent to which the association is due to confounding by indication.[453]

The authors searched OVID for Medline, Embase, and PsycINFO, and EBSCO for CINAHL, from inception to August 18, 2022. The authors included all peer-reviewed, English-language studies on in utero APAP exposure and child neurodevelopmental outcomes. Data were extracted using a standardized form created a priori. The quality of the studies was assessed using the Systematic Assessment of Quality in Observational Research. The authors generated pooled risk ratios (RR) for outcomes examined by ≥3

[452] Patel et al. Long-Term Safety of Prenatal and Neonatal Exposure to Paracetamol: A Systematic Review. Int J Environ Res Public Health. 2022 Feb 14;19(4):2128. doi: 10.3390/ijerph19042128. PMID: 35206317; PMCID: PMC8871754.
[453] Ricci et al. In utero acetaminophen exposure and child neurodevelopmental outcomes: Systematic review and meta-analysis. Paediatr Perinat Epidemiol. 2023 Mar 20. doi: 10.1111/ppe.12963. Epub ahead of print. PMID: 36939050.

studies using random-effects models; outcomes that could not be meta-analyzed were narratively summarized following Synthesis Without Meta-Analysis guidelines.

The quality assessments resulted in 13.6% of studies being classified as high, 59.1% as medium, 22.7% as low, and 4.5% as very low quality. The study found that *in utero* APAP exposure was associated with an elevated risk of ADHD (unadjusted pooled risk ratio 1.32, 95% confidence interval (1.20-1.44); I^2 = 47%, n = 7 studies), and the association remained after adjusting for confounders, including indications for APAP use (adjusted pooled risk ratio 1.34, 95% confidence interval (1.15-1.55); I^2 = 50%, n = 4 studies). The I^2 statistic is used to assess the degree of heterogeneity or variability across studies included in the analysis. The reported 47-50% would be considered moderate heterogeneity. It indicates that there is some heterogeneity between studies beyond what can be attributed to chance alone.

In conclusion, this systematic review and meta-analysis reported a significant association between in utero APAP exposure and development of ADHD that was not explained by confounding by indication.

Masarwa 2018

This meta-analysis aimed to assess the role of potential unmeasured confounding in the estimation of the association between acetaminophen use during pregnancy and the risk of attention deficit hyperactivity disorder (ADHD) in offspring.[454] The study included seven observational studies that examined this association. The results showed that when adjusted estimates were pooled across all studies, the risk ratio (RR) for ADHD was 1.35 (95% confidence interval [CI] 1.25, 1.46; I2 = 48%). Sensitivity analysis for unmeasured confounding showed that a confounder of 1.69 on the RR scale would reduce to 10% the proportion of studies with a true effect size of RR >1.10.

The conclusion of the meta-analysis is that bias analysis suggests that the previously reported association between acetaminophen use during pregnancy and an increased risk of ADHD in offspring may be due to unmeasured confounding, and that our ability to conclude a causal association is limited.

Masarwa 2020

This study aimed to assess the role of potential unmeasured confounding in the estimation of the association between acetaminophen use during pregnancy and the risk of attention deficit hyperactivity ADHD in offspring, through bias analysis.[455] Bias analysis involves identifying and assessing the potential sources of bias in a study and evaluating their potential impact on the study results. The goal is to understand how biases may have influenced the observed associations and to estimate the direction and magnitude of the bias. The secondary objective was to assess the roles of selection bias and exposure misclassification.

The study conducted a systematic review and meta-analysis of observational studies examining the association between acetaminophen use during pregnancy and the risk of ADHD in offspring. The authors searched PubMed, Embase, and Web of Science for relevant studies published up to December 2019. The authors included cohort and case-control studies that reported on the association between APAP use

[454] Masarwa et al. Prenatal Exposure to Acetaminophen and Risk for Attention Deficit Hyperactivity Disorder and Autistic Spectrum Disorder: A Systematic Review, Meta-Analysis, and Meta-Regression Analysis of Cohort Studies. Am J Epidemiol. 2018 Aug 1;187(8):1817-1827. doi: 10.1093/aje/kwy086. PMID: 29688261.

[455] Masarwa et al. Acetaminophen use during pregnancy and the risk of attention deficit hyperactivity disorder: A causal association or bias? Paediatr Perinat Epidemiol. 2020 May;34(3):309-317. doi: 10.1111/ppe.12615. Epub 2020 Jan 9. PMID: 31916282.

during pregnancy and the risk of ADHD in offspring. Studies were excluded if they did not report on APAP exposure during pregnancy or if they did not report on ADHD as an outcome. Two reviewers independently extracted data from the included studies, including study characteristics, exposure and outcome definitions, and results. Discrepancies were resolved through discussion. The authors assessed the quality of the included studies using the Newcastle-Ottawa Scale for cohort and case-control studies.

The authors conducted a random-effects meta-analysis to pool adjusted risk estimates across studies. They also conducted a bias analysis to assess the potential impact of unmeasured confounding, selection bias, and exposure misclassification on the results. The meta-analysis included seven studies with a total of 132,738 mother-child pairs. When adjusted estimates were pooled across all studies, the risk ratio (RR) for ADHD was 1.35 (95% confidence interval (CI), 1.25-1.46; $I^2 = 48\%$). However, bias analysis suggests that the previously reported association between APAP use during pregnancy and an increased risk of ADHD in offspring may be due to unmeasured confounding.

The study concludes that their ability to conclude a causal association between APAP use during pregnancy and an increased risk of ADHD in offspring is limited due to potential unmeasured confounding. Unmeasured confounding occurs when a variable that is associated with both the exposure and the outcome is not measured or controlled for in the study.

Weight of the Evidence for Meta-Analyses Investigating the Association Between APAP and ASD and ADHD.

Weight of evidence analysis is a systematic approach to evaluating the strength of scientific evidence by considering the quality, quantity, and consistency of the available data. A meta-analysis is a statistical analysis that combines the results of multiple scientific studies to produce a single estimate of the effect of an intervention or exposure. Using the hierarchy of evidence approach, meta-analyses are reported to provide level 1 evidence, see Figure 1, as they synthesize the results of multiple studies on a specific topic. However, not all meta-analyses are equally reliable, and they may vary in their methods, quality, and conclusions. Therefore, it is important to critically appraise the meta-analyses and assess their strengths and limitations. Here is a summary of the findings and odds ratios reported by each meta-analysis in the current web page context, followed by a conclusion based on the evidence:

Alemany 2021: This meta-analysis found that maternal use of APAP during pregnancy was associated with an increased risk of ADHD (pooled relative risk [RR] = 1.29, 95% confidence interval [CI] = 1.20–1.39) and ASD (pooled RR = 1.21, 95% CI = 1.08–1.36) in offspring. The association was stronger for male offspring than for female offspring. The meta-analysis included 6 European Cohort studies with a total of 73,881mother-child pairs.

Gou 2019: This meta-analysis found that maternal use of APAP during pregnancy increased the risk of ADHD in offspring with a pooled adjusted risk ratio of 1.25 (95% CI = 1.17-1.34). The risk was higher for children exposed to APAP in the third trimester and for longer duration of use. The meta-analysis included eight studies with a total of 244,940 participants.

Kim 2019: This umbrella review of meta-analyses found that APAP use during pregnancy was one of the environmental risk factors that had highly suggestive evidence (class II) of association with ASD. The random effects summary estimate for this association was a relative risk of 1.20 (95% CI 1.14–1.26) based

on five cohort studies with more than 1000 cases of ASD. However, this association showed signs of small study effects and was not significant under 10% credibility ceiling, meaning that it could be affected by bias or confounding factors.

Kim 2020: This umbrella review of meta-analyses found that APAP use during pregnancy had convincing evidence (class I) of association with ADHD. The random effects summary estimate for this association was a relative risk of 1.25 (95% CI 1.17 to 1.34) based on seven studies with a total of 132,738 mother-child pairs. However, the authors noted that there was a possibility of residual confounding, as not all potential confounders were adjusted for in the included studies.

Patel 2022: This systematic review found that prenatal APAP exposure was associated with an increased risk of neurodevelopmental, atopic and reproductive adverse outcomes in children, such as asthma, wheezing, eczema, behavior problems, ADHD, ASD, reduced anogenital distance and delayed pubertal development. The review included 30 studies, most of which were observational epidemiologic and cohort studies.

Ricci 2023: This systematic review and meta-analysis found that in utero APAP exposure was associated with an elevated risk of ADHD (unadjusted pooled RR 1.32, 95% CI 1.20-1.44; $I^2 = 47\%$, n = 7 studies), and the association remained after adjusting for confounders, including indications for APAP use (adjusted pooled RR 1.34, 95% CI 1.15-1.55; $I^2 = 50\%$, n = 4 studies). The meta-analysis included 22 studies with a total of 367,775 participants.

Masarwa 2018: This meta-analysis found that when adjusted estimates were pooled across all studies, the RR for ADHD was 1.35 (95% CI 1.25, 1.46; I2 = 48%). However, bias analysis suggested that the previously reported association between APAP use during pregnancy and an increased risk of ADHD in offspring may be due to unmeasured confounding, and that the ability to conclude a causal association is limited. The meta-analysis included seven studies with a total of 132,738 mother-child pairs.

Masarwa 2020: This study conducted a bias analysis to assess the potential impact of unmeasured confounding, selection bias, and exposure misclassification on the results of the previous meta-analysis by Masarwa et al., 2018. The study confirmed that the observed association between APAP use during pregnancy and an increased risk of ADHD in offspring may be due to unmeasured confounding.

Based on the hierarchy of evidence approach, most of the meta-analyses provide high-quality evidence that maternal use of APAP during pregnancy is associated with an increased risk of NDDs, including ADHD and ASD, in offspring.

Overall, the weight of evidence indicates **APAP has "clear evidence" of developmental toxicity**, based on the consistent finding in multiple meta-analyses. **There are moderate associations between maternal use of APAP during pregnancy and an increased risk of ADHD and ASD in offspring.** These reviewed meta-analyses consistently confirmed and reported associations between APAP and ASD and ADHD, including dose-response by dose or duration.

E. Other Relevant Reviews of the Literature on APAP and Brain Development

Bauer 2021

This review and consensus statement summarizes the evidence on prenatal exposure to APAP (also referenced as paracetamol/acetaminophen) altering fetal development and increasing the risks of certain neurodevelopmental, reproductive, and urogenital disorders. They call for precautionary action through focused research efforts and by increasing awareness among health professionals and pregnant women.

The authors recommend that pregnant women should be cautioned at the beginning of pregnancy to forego APAP unless its use is medically indicated; consult with a physician or pharmacist if they are uncertain whether use is indicated and before using on a long-term basis; and minimize exposure by using the lowest effective dose for the shortest possible time. They suggest specific actions to implement these recommendations.

This consensus statement reflects the concerns of the authors and was signed by 91 scientists, clinicians, and public health professionals from across the globe. The authors acknowledge that APAP is an important medication and alternatives for treatment of high fever and severe pain are limited. However, they believe that taking a precautionary approach is warranted given the growing body of experimental and epidemiological research suggesting potential risks associated with prenatal exposure to APAP.

Buhrer 2021

This article discusses the growing concerns that APAP use during pregnancy may cause attention deficit hyperactivity disorder (ADHD) and autism spectrum disorder (ASD) in the offspring. It reports a growing number of epidemiological studies suggest that relative risks for these disorders increase by an average of about 25% following intrauterine paracetamol exposure. The data analyzed point to a dose-effect relationship but cannot fully account for unmeasured confounders, notably indication and genetic transmission.

The study also summarizes the altered behavior reported in offspring of paracetamol-treated pregnant rats, and paracetamol given at or prior to day 10 (PND10) in newborn mice resulted in altered locomotor activity in response to a novel home environment in adulthood and blunted the analgesic effect of paracetamol given to adult animals.

The study concludes that given the widespread use of paracetamol during pregnancy and the lack of safe alternatives, its impact on the developing brain deserves further investigation. Caution is warranted when considering long-term use of paracetamol during pregnancy; however, women with severe pain conditions should not be deprived of appropriate treatments.

Cendejas-Hernandez 2022

This study is a systematic review that aimed to assess the safety of APAP use in infants and children for neurodevelopment.[456] The authors searched PubMed, Embase, and Web of Science for relevant studies published up to December 2019. The authors included studies that reported on the association between APAP use in infants and children and neurodevelopmental outcomes. The authors extracted data from the included studies, including study characteristics, exposure and outcome definitions, and results. The

[456] Cendejas-Hernandez et al. Paracetamol (acetaminophen) use in infants and children was never shown to be safe for neurodevelopment: a systematic review with citation tracking. Eur J Pediatr. 2022 May;181(5):1835-1857. doi: 10.1007/s00431-022-04407-w. Epub 2022 Feb 17. PMID: 35175416; PMCID: PMC9056471.

authors conducted a systematic review with citation tracking to assess the evidence on the safety of APAP use in infants and children for neurodevelopment.

The study concludes that although widely believed by pediatricians and parents to be safe for use in infants and children when used as directed, increasing evidence indicates that early life exposure to APAP may cause long-term neurodevelopmental problems.

Havdahl 2022

The study used data from the Norwegian Mother, Father and Child Cohort Study (MoBa) to assess parental genetic liability to attention-deficit/hyperactivity disorder (ADHD), autism, and schizophrenia.

Outcome definition and determination: The outcomes examined were offspring neurodevelopmental conditions, including ADHD, autism, and schizophrenia.

Control group: The control group would be offspring without neurodevelopmental conditions.

Study size: The study included data from the MoBa cohort, which recruited pregnant women in Norway between 1999 and 2008.

Confounding factors or biases and how or if they were controlled: The study adjusted for a number of factors, including maternal age, education, smoking, alcohol use, pre-pregnancy body mass index (BMI), parity, marital status, household income, life events during pregnancy, symptoms of depression/anxiety during pregnancy, use of other pain medication during pregnancy, fever/infection during pregnancy, child sex, gestational age at birth, birth weight, and breastfeeding duration.

Overall, the study reported that genetic liability to neurodevelopmental conditions that is passed from mothers to children was associated with several pregnancy-related factors and may therefore confound associations between these pregnancy-related factors and offspring neurodevelopment that have previously been thought to be causal. The study concluded that further research is needed to clarify these associations.

F. Prenatal APAP Exposure and Other Outcomes

In this section, I review studies investigating prenatal exposure to APAP and birth outcomes other than impaired learning, cognitive, or social behavior.

Gervin 2017

This is a cohort study design using samples selected from the Norwegian Mother and Child Cohort (MoBa).[457]

[457] Gervin et al. Long-term prenatal exposure to paracetamol is associated with DNA methylation differences in children diagnosed with ADHD. Clin Epigenetics. 2017 Aug 2;9:77. doi: 10.1186/s13148-017-0376-9. PMID: 28785368; PMCID: PMC5540511.

Information used to determine exposure: Information about paracetamol use was based on three questionnaires completed during pregnancy. Long-term use was defined as use of paracetamol for 20 days or more during pregnancy.

Outcome definition and determination: The outcome of interest was DNA methylation differences associated with prenatal exposure to paracetamol and ADHD in cord blood samples. ADHD diagnoses were obtained from the Norwegian Patient Registry (NPR) from 2008 to 2014.

Control group: The control group consisted of 96 samples that were unexposed to paracetamol and without ADHD.

Study size: The study included a total of 384 samples, with 96 samples in each of the four categories: unexposed to paracetamol without ADHD, exposed to paracetamol without ADHD, unexposed to paracetamol with ADHD, and exposed to paracetamol with ADHD.

Confounding factors or biases and how or if they were controlled: Potential covariates such as infant sex, gestational age at delivery, maternal age, smoking, and alcohol consumption during pregnancy were included in the statistical model. The study also corrected for cord blood cell-type composition between groups using a suitable cord blood reference data set.

Results: The study reported that prenatal exposure to paracetamol/acetaminophen and its precursor aniline impaired masculinization of the male brain and behavior.

The study concludes that their findings support that prenatal exposure to APAP and its precursor aniline impairs masculinization of the male brain and behavior, and that in susceptible individuals, prenatal long-term exposure to APAP is associated with DNA methylation in genes involved in oxidative stress.

Eslamimehr 2022

This study is a cohort study that examined the association between prenatal APAP use and acetaminophen metabolites with DNA methylation of newborns.[458]

Exposure: The exposure of interest in this study was prenatal APAP use and APAP metabolites. Information on prenatal acetaminophen use was obtained from maternal self-reported questionnaires. This is a study limitation, because self-reported maternal APAP use during pregnancy may be subject to recall bias or measurement error. Acetaminophen metabolites were also measured in maternal urine samples collected during pregnancy, which mitigates this limitation.

Outcome definition and determination: The outcome of interest in this study was DNA methylation of newborns. DNA methylation was measured in cord blood samples collected at birth. DNA, cytosine-phosphate-guanine (CpG) CpG, dinucleotides were examined. CpG islands refer to a specific DNA sequence region that contains a high frequency of CpG dinucleotides. CpG islands are characterized by having a high density of CpG sites, which are usually methylated. Methylation refers to the addition of a

[458] Eslamimehr et al. Association of prenatal acetaminophen use and acetaminophen metabolites with DNA methylation of newborns: analysis of two consecutive generations of the Isle of Wight birth cohort. Environ Epigenet. 2022 Feb 2;8(1):dvac002. doi: 10.1093/eep/dvac002. PMID: 35317219; PMCID: PMC8933617.

methyl group (CH3) to the cytosine nucleotide of a CpG dinucleotide. Methylation of CpG islands in gene promoters is associated with gene silencing or reduced gene expression. In general, the presence of a CpG island near a gene promoter region is associated with active transcription and gene expression, while methylation of CpG sites within the island can lead to gene repression.

Control group: This study did not have a traditional control group as it was a cohort study following pregnancies among women who did not report using acetaminophen during pregnancy.

Study size: The study included 1,056 mother-child pairs from two consecutive generations of the Isle of Wight Birth Cohort.

Confounding factors or biases and how or if they were controlled: The study adjusted for several potential confounding factors, including maternal age, pre-pregnancy body mass index (BMI), smoking status, alcohol consumption, parity, education level, and child sex. Additionally, the study used propensity score matching to control for potential confounding by indication.

Results: The study found that 21 differential DNA methylation sites in cord blood were associated with reported maternal acetaminophen intake in the F2 generation. For 11 of these CpG sites, an indication bias was excluded and five were replicated in F2 with metabolite clusters. In addition, metabolite clusters showed associations with 25 CpGs in the F0-F1 discovery analysis, of which five CpGs were replicated in the F2-generation.

The study concludes that these findings support that prenatal APAP use has an impact on the epigenetic profile of newborns. This is important because DNA methylation is a mechanism by which environmental exposures can influence gene expression and potentially impact health outcomes later in life.

Arneja 2020

The study is a prospective pregnancy and birth cohort study.[459]

Information used to determine exposure: Use of over-the-counter (OTC) analgesics, including acetaminophen, was identified from questionnaires. Exposure was categorized by timing of use: (i) prepregnancy: in the 3 months before pregnancy, (ii) early pregnancy: in the first 12–16 weeks of pregnancy, and (iii) mid–late pregnancy: between completion of the first questionnaire and 28–32 weeks of gestation. Acetaminophen use was also categorized by frequency into: never, less than once per week, and one or more times per week.

Outcome definition and determination: The outcomes examined were preterm birth, low birthweight, and small for gestational age. Information on the baby's sex, birthweight, and gestational age was derived from clinical data collected from hospital medical charts.

Control group: The control group would be women who never used acetaminophen.

Study size: The study included 1,200 women within the Ontario Birth Study who delivered between January 2013 and June 2017.

[459] Arneja et al. Association between maternal acetaminophen use and adverse birth outcomes in a pregnancy and birth cohort. Pediatr Res. 2020 Jun;87(7):1263-1269. doi: 10.1038/s41390-019-0726-8. Epub 2019 Dec 18. PMID: 31852009.

Confounding factors or biases and how or if they were controlled: Covariates included a priori defined variables based on literature review and hypothesized relationships between acetaminophen use and birth outcomes. Information on covariates was obtained from lifestyle questionnaires. Multivariable adjusted models included age, smoking in the 3 months before pregnancy, body mass index (BMI) at baseline, maternal ethnicity, education, fever during pregnancy, paternal smoking in the 3 months before pregnancy, a comorbidity index created as a sum of the number of pre-existing conditions reported by each individual, and participants' use of other pain medications including OTC nonsteroidal anti-inflammatory drugs and prescription pain medications such as diclofenac, morphine, oxycodone, and codeine during the respective time periods.

Results: the study found that children born to mothers who used acetaminophen in the 3 months before pregnancy had a higher risk of having low birthweight and being born small for gestational age. Further, results presented evidence of dose response with more frequent use of acetaminophen being associated with increased risk of the offspring being born small for gestational age. During pregnancy, however, maternal use of APAP was not associated with the examined adverse birth outcomes: preterm birth, low birthweight, and small for gestational age. In addition, continuous use of acetaminophen (relative to never use) was not associated with any of the examined birth outcomes.

The study concluded that pre-pregnancy APAP use is associated with higher risk of adverse birth outcomes.

Baker 2022

This was a prospective cohort study.[460]

Information used to determine exposure: APAP exposure was determined by measuring APAP levels in meconium samples collected at birth.

Outcome definition and determination: The outcomes of interest were birthweight, gestational age, preterm birth, size for gestational age, gestational diabetes, preeclampsia, and high blood pressure. These outcomes were determined using medical records and standardized definitions.

Control group: The control group consisted of infants with no detectable levels of APAP in their meconium samples.

Study size: The study included 1,062 mother-infant pairs.

Confounding factors or biases and how or if they were controlled: The study controlled for potential confounding factors such as maternal age, pre-pregnancy BMI, smoking status, alcohol consumption, and socioeconomic status using multivariable regression models.

[460] Baker et al. Association of Prenatal Acetaminophen Exposure Measured in Meconium With Adverse Birth Outcomes in a Canadian Birth Cohort. Front Pediatr. 2022 Apr 5;10:828089. doi: 10.3389/fped.2022.828089. PMID: 35450103; PMCID: PMC9017809.

Limitations: (1) The homogeneity of the GESTE cohort limits the generalizability of results despite minimizing confounding. Variations in the association of meconium APAP with birthweight were observed between individuals recruited during pregnancy and at delivery, possibly due to different distributions of unmeasured genetic and environmental modifiers in each group. (2) The study had a small sample size of nearly 400 mother-child pairs, resulting in few events for certain outcomes such as early prematurity and severe preeclampsia. (3) The exclusion of extremely preterm deliveries may introduce selection bias and limit conclusions about preterm birth. (4) The study lacked information to control for indications of APAP use during pregnancy, potentially leading to confounding by indication. (5) The detection of APAP in meconium may be influenced by the size of infants, but inverse causality is unlikely. (6) Self-reported APAP use and correlating it with meconium levels were not conducted, limiting knowledge of dosage and timing of exposure. (7) Meconium may have limited utility for studying severe prematurity and early pregnancy outcomes since it forms in later stages of pregnancy. (8) The long-term storage of meconium samples at −80°C raises the possibility of sample degradation, although the stability of APAP in meconium under these conditions is unknown.

Results: The results indicated that prenatal acetaminophen exposure was linked to a 136 g decrease in birthweight, a 20% higher weekly delivery hazard, and a more than 60% reduced likelihood of being born large for gestational age. No associations were found between prenatal APAP use and small for gestational age, preterm birth, or any pregnancy complications.

Vedel 2022

The study was a qualitative study based on a short questionnaire.[461]

Information used to determine exposure: The study used self-reported data from pregnant women who completed a questionnaire regarding their use of APAP during pregnancy. This is a study limitation, because self-reported maternal APAP use during pregnancy may be subject to recall bias or measurement error.

Outcome definition and determination: The outcome of the study was to investigate the feasibility of executing a randomized clinical trial (RCT) in light of the results of a new qualitative study on APAP use during pregnancy.

Control group: Not applicable as this was a qualitative study.

Study size: A total of 232 pregnant women completed the questionnaire with a mean gestational age of 13 weeks and 6 days.

Confounding factors or biases and how or if they were controlled: Not applicable as this was a qualitative study.

Results: The study reported that 48% of the women had taken APAP after recognizing their pregnancy, and only 6% had taken APAP weekly or more than weekly. Overall, 34% would participate in an RCT regarding APAP and 49% would not. The rest were in doubt. Of the women who had taken APAP after

[461] Vedel et al. The use of paracetamol during pregnancy: A qualitative study and possible strategies for a clinical trial. PLoS One. 2022 Sep 13;17(9):e0271537. doi: 10.1371/journal.pone.0271537. PMID: 36099269; PMCID: PMC9469947.

becoming pregnant, 44% would not participate in an RCT. That leaves a total of 27% who had taken any APAP in the first trimester of pregnancy that would potentially participate in an RCT.

G. Conclusions Based on WoE of Epidemiological Studies

As reviewed above, the developing brain is vulnerability to oxidative damage and neurotoxicity during the Brain Growth Spurt (BGS). In humans, the BGS peaks at around birth but continues during early post-natal development. As a result, the vulnerability of the fetal brain during the third-trimester to the toxic effects of APAP is also present in the infant brain in the weeks after birth. As a result, infant exposure to APAP can also cause ASD and ADHD. In an epidemiological study limited to APAP exposure during pregnancy, any ASD or ADHD caused by infant exposure to APAP postnatally could be a significant factor that would lower the reported odds ratio for *in utero* associations between APAP and ASD and ADHD. This misclassification was raised as a concern in several studies, as self-reporting was the primary mode of determining APAP exposures. Studies that measured APAP do not support such misclassification or confounding by indication. For example, the study from Ji et al. 2020 that relied on measurements of APAP in cord plasma reported that children in the second tertile (OR = 2.14; CI 0.93-5.13) and third tertile (OR = 3.62; CI 1.62-8.60) had a higher odds of an ASD diagnosis compared to studies that relied on maternal self-reporting. This suggests that studies based on maternal self-reporting are biased towards the null, and that the actual risks of ASD are higher than reported in such studies.

In addition, certain factors identified as potential confounders that are controlled for in a study can be mediators or modifiers of toxicity. For example, a sibling control study would mask the effects of a genetic vulnerability (e.g., a deficiency in glutathione synthesis or transport) that is present in siblings. Such a genetic vulnerability may not cause ASD or ADHD without additional environmental stress. In such a case, genetic or nutritional modifiers are not an alternative causal path, they are within the same oxidative stress AOP. Regarding familial and sibling interactions, Dr. Baccarelli's report includes a cogent explanation of how a sibling-control study would mask or mitigate the association between APAP and ASD or ADHD.

Independent review of the available epidemiological evidence indicates "some evidence" for APAP exposure causing ADHD or ASD. The majority of these studies are limited because of self-reporting. The conclusions based on available meta-analyses are that maternal use of APAP during pregnancy provides "clear evidence" of increased risk of ADHD and ASD in offspring. These data consistently show associations between APAP and ASD and ADHD, including dose-response by dose or duration. Two meta-analyses, both by the same group/first author, do present "unmeasured confounding" as an alternative explanation. Three studies analyzed exposure via biological fluids, including cord blood and meconium. Of the three studies analyzing biological fluids, two of the three demonstrated a dose-responsive increase in ADHD or ASD risk with higher levels of APAP exposure. Collectively, these studies provide "clear evidence" for APAP exposure causing ADHD and ASD.

I have also reviewed the Expert Report of Dr. Andrea Baccarelli that addresses the epidemiological evidence, and I concur with his analysis and conclusions.

Based on the hierarchy of evidence approach, most of the meta-analyses provide high-quality evidence that maternal use of APAP during pregnancy is associated with an increased risk of NDDs, including ADHD and ASD, in offspring.

Overall, the weight of evidence indicates **APAP has "clear evidence" of developmental toxicity**, based on the consistent finding on increased risk of ASD and ADHD in multiple meta-analyses. **There are**

moderate associations between maternal use of APAP during pregnancy and an increased risk of ADHD and ASD in offspring. These reviewed meta-analyses consistently confirmed and reported associations between APAP and ASD and ADHD, including dose-response by dose or duration.

AUTISM GENETICS & EPIGENETICS

A key concept in teratology is that the majority of structural birth defects are caused by the combined effects of individual host susceptibility (genetic factors) that interact with exogenous exposures (environmental factors) in other words, gene-environmental interactions. Such interactions or effect modification can arise between a given gene or collection of genes, and various environmental factors. The complexity of such interactions can quickly become challenging to elucidate analytically and to test experimentally in a rigorous hypothesis-driven manner. Does the genetic factor exacerbate the environmental factor (or vice versa) to increase overall risk for the observed birth defect? Alternatively, are both the genetic factor and the environmental factor necessary to increase the defect risk? Additional questions can also arise as to whose genetic background (mother, father, or infant) and whose exposure (mother or father) is most pertinent. As with other observational studies of human disorders, identification of risks between a gene and an environmental factor requires a careful assessment of whether the observed relationship has arisen by chance or is the result of some bias. For example, has some selective force in the study design or methods allowed an excess of cases (or a deficiency of controls) with both the genetic and the environmental factor(s)?

Autism spectrum disorder (ASD) is a complex neurodevelopmental disorder with a multifactorial etiology, that includes genetic and environmental factors. ASD affects nearly 2% of children in the United States, and both genetic and environmental factors are proposed to be involved in the etiology. No single gene has been identified as the cause of ASD, but genetics are considered a factor in the etiology of autism with hundreds of genes being implicated in its pathogenesis.

Attention deficit hyperactivity disorder (ADHD) is a neurodevelopmental disorder that is reported to affect 3-6% of school-age children and shows evidence of familial inheritance. ADHD is characterized by symptoms of inattention, hyperactivity, and impulsivity. Like ASD, ADHD is a complex disorder that is influenced by both genetic and environmental factors. While no single gene has been identified as the sole cause of ADHD, several genes have been associated with the disorder.

Genetic studies report that ASD and ADHD are not solely the result of genetic factors and that the rise in the prevalence of the diseases cannot be explained by genetics alone. Studies also demonstrate that APAP targets a large number of genes identified as making someone susceptible to ASD.

Carter and Blizard examined environmental pollutants including pesticides, heavy metals, bisphenol A, phthalates and many others in food, cosmetics or household products for potential roles of environmental pollutants in the etiology of Autism Spectrum Disorder (ASD).[462] The study analyzed gene/environment interactions in autism using 206 autism susceptibility genes (ASGs) from the Autworks database to interrogate ~1 million chemical/gene interactions in the comparative toxicogenomics database. The study found that a number of compounds identified in epidemiology selectively target autism genes, including

[462] Carter and Blizard. Autism genes are selectively targeted by environmental pollutants including pesticides, heavy metals, bisphenol A, phthalates and many others in food, cosmetics or household products. Neurochem Int. 2016 Oct 27:S0197-0186(16)30197-8. doi: 10.1016/j.neuint.2016.10.011. Epub ahead of print. PMID: 27984170.

APAP. The study authors found that the number of autism susceptibility genes targeted by APAP (9,176) exceeded all other tested compounds, except for Valproic Acid (VPA), a compound that is used to induce and model ASD symptoms in rodents.

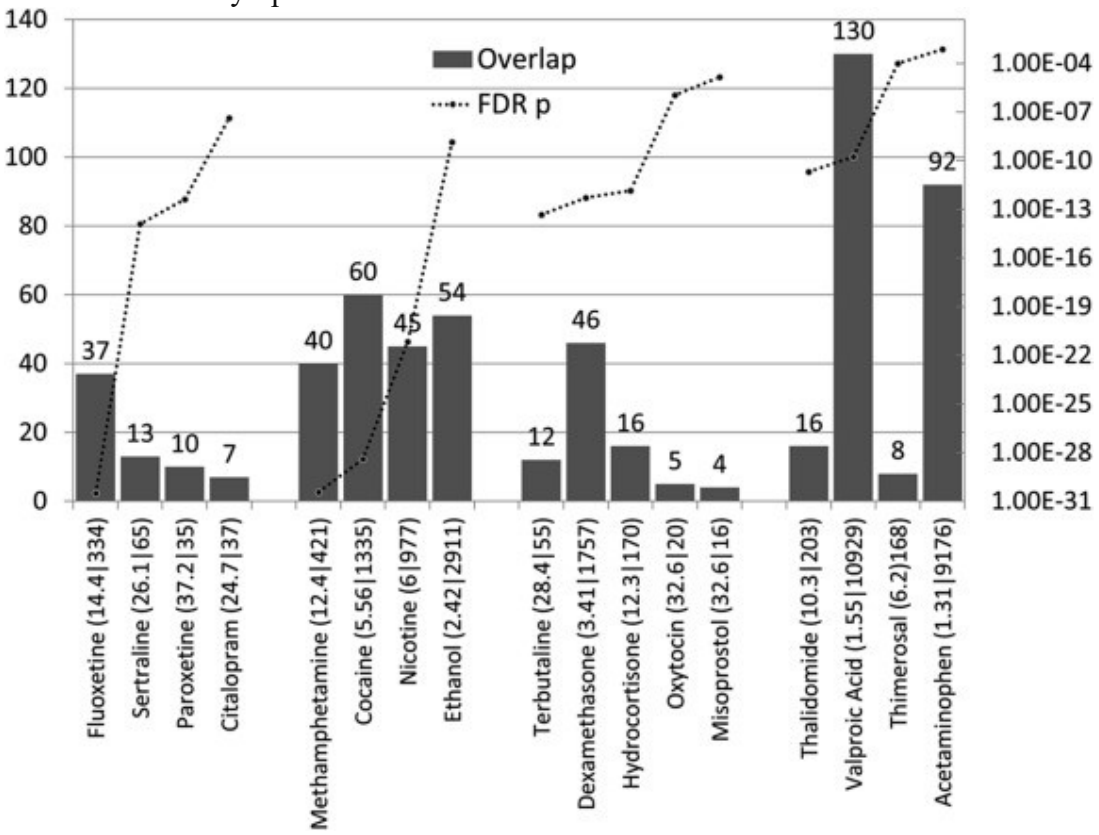


Figure 33. The Number of Autism Susceptibility Genes (ASGs). Reported ASGs are affected by diverse endocrine disruptors. (Number of ASGs = left axis, p values = right axis). The enrichment ratio and the total number of genes affected by each compound are shown after each compound name. Acetaminophen is shown in the last bar, with significant enrichment of ASGs.

Santos et al. discusses the potential role of gene-environment interactions in the etiology of ASD.[463] The study aimed to identify genes involved in the regulation of xenobiotic detoxification or the function of physiological barriers (the XenoReg genes) presenting predicted damaging variants in subjects with ASD and to understand their interaction patterns with ubiquitous xenobiotics previously implicated in this disorder. The study defined a panel of 519 XenoReg genes through literature review and database queries and inspected large ASD datasets for predicted damaging Single Nucleotide Variants (SNVs) or Copy Number Variants (CNVs) in XenoReg genes. The interrogation of ASD datasets for variants in the XenoReg gene panel identified 77 genes with high evidence for a role in ASD, according to pre-specified prioritization criteria. These include 47 genes encoding detoxification enzymes and 30 genes encoding proteins involved in physiological barrier function, among which 15 are previous reported candidates for ASD. The study concludes that individuals carrying predicted damaging variants in high evidence XenoReg genes are likely to have less efficient detoxification systems or impaired physiological barriers and can therefore be particularly susceptible to early life exposure to ubiquitous xenobiotics, which elicit

[463] Santos et al. A Role for Gene-Environment Interactions in Autism Spectrum Disorder Is Supported by Variants in Genes Regulating the Effects of Exposure to Xenobiotics. Front Neurosci. 2022 May 19;16:862315. doi: 10.3389/fnins.2022.862315. PMID: 35663546; PMCID: PMC9161282.

neuropathological mechanisms in the immature brain. As exposure to environmental factors may be mitigated for individuals with risk variants, this work provides new perspectives to personalized prevention and health management policies for ASD.

There is no single gene implicated in the etiology of ADHD, but various gene mutations have been associated with ADHD, including:

DRD4: This gene encodes the dopamine receptor D4, which is involved in the regulation of attention, motivation, and reward. Variations in this gene have been associated with an increased risk of ADHD.[464, 465]

DAT1 / SLC6A3: This gene encodes the dopamine transporter, which is involved in the regulation of dopamine levels in the brain. Variations in this gene have been associated with an increased risk of ADHD.[466, 467, 468]

Epigenetics

Epigenetics concerns itself with the manner in which environmental factors, working through chemical signals or proteins modify the expression of genes without changing the structure of DNA (deoxyribonucleic acid). The nucleic acid chain that makes up the genes in the cell's nucleus is DNA. The messenger molecule that carries information from the nucleus out to the cytoplasm of the cell is RNA (ribonucleic acid). The capacity of various environmental toxicants to disrupt the function of these nucleic acids, or to destroy them outright, has an important bearing on causation and mechanism.

It is now well established that environmental exposures during the first 1000 days post-conception can result in epigenetic imprints that are transmissible to the baby and beyond to subsequent generations. Some of the environmental risk factors which shape the genetic and epigenetic signature of the offspring include: maternal nutritional status and stress, xenobiotics, recreational exposures (alcohol, drugs and smoking) as well as maternal occupational contaminants.[469,470] Early life is a critical period during which the differentiation process leading to specialized cells from the pluripotent cells, is mediated by 'epigenetic remodeling' such that genes that do not have to be expressed in a particular tissue type are turned off, while maintaining active gene expression for genes in those tissues for which it is appropriate to remain

[464] LaHoste Get al. Dopamine D4 receptor gene polymorphism is associated with attention deficit hyperactivity disorder. Mol Psychiatry. 1996 May;1(2):121-4. PMID: 9118321.
[465] Grady et al. High prevalence of rare dopamine receptor D4 alleles in children diagnosed with attention-deficit hyperactivity disorder. Mol Psychiatry. 2003 May;8(5):536-45. doi: 10.1038/sj.mp.4001350. PMID: 12808433.
[466] Cook et al. Association of attention-deficit disorder and the dopamine transporter gene. Am J Hum Genet. 1995 Apr;56(4):993-8. PMID: 7717410; PMCID: PMC1801209.
[467] Gill et al. Confirmation of association between attention deficit hyperactivity disorder and a dopamine transporter polymorphism. Mol Psychiatry. 1997 Jul;2(4):311-3. doi: 10.1038/sj.mp.4000290. PMID: 9246671.
[468] Daly et al. Mapping susceptibility loci in attention deficit hyperactivity disorder: preferential transmission of parental alleles at DAT1, DBH and DRD5 to affected children. Mol Psychiatry. 1999 Mar;4(2):192-6. doi: 10.1038/sj.mp.4000510. PMID: 10208453.
[469] Hanson and Gluckman. Early developmental conditioning of later health and disease: physiology or pathophysiology? Physiol Rev. 2014 Oct;94(4):1027-76. doi: 10.1152/physrev.00029.2013. PMID: 25287859; PMCID: PMC4187033.
[470] Burton and Metcalfe. Can environmental conditions experienced in early life influence future generations? Proc Biol Sci. 2014 May 7;281(1785):20140311. doi: 10.1098/rspb.2014.0311. PMID: 24807254; PMCID: PMC4024293.

active.[471,472] In this context, epigenetic alterations via DNA methylation and post-translational modifications work to differentiate cells properly. DNA methylation depends on the activity of specific enzymes called DNA methyltransferases (DNMTs), which promotes the methylation (the addition of CH_3 groups) to specific DNA nucleotides within a region of a gene referred to as CpG islands, at the promoter of a gene leading to a progressive switching off of the gene. Anything that inhibits the interaction between chemical signaling proteins called transcription factors and the promoter region of a gene due to the presence of methyl groups (CH_3) limits their binding with RNA-polymerases that are required to turn on gene expression.[473] At the same time, methylation of regulatory regions within any given gene, serves as an additional control of gene expression.

As the term implies, epigenetics represents a level of genetic regulation that is not limited to the messages coded in the DNA sequence. Genomic mutational catalogs are now available for numerous cancers, producing databases such as COSMIC: the Catalogue Of Somatic Mutations In Cancer.[474] Analysis of this data has produced novel mutational signatures associated with chemical exposures, such as smoking.[475] Epigenetic fingerprints are also emerging and are associated with specific chemical exposures.[476] In both genetics and epigenetics, signatures emerge because some regions of the genome are more susceptible to specific chemicals than others, producing genetic and epigenetic/epigenomic alterations characteristic of environmental factors. There has been progress in analytical tools and interpretation of genomic architecture, but our understanding of genomics and epigenomics is by no means complete. While telomere-to-telomere genomics is filling in genomic gaps and becoming more available,[477] there is still more information needed before the epigenetic regulation of all protein coding genes is properly interrogated and understood.[478]

LaSalle reviewed epigenetic signatures for ASD.[479] The review discusses how epigenetic investigations, including DNA methylation, have emerged as a novel way to capture the complex interface of multivariate autism spectrum disorder (ASD) etiologies. Epigenome-wide association studies using human brain and surrogate accessible tissues have revealed some convergent genes that are epigenetically altered in ASD, many of which overlap with known genetic risk factors. Epigenomic signatures defined by DNA methylation from surrogate tissues such as placenta and cord blood can reflect past differences in fetal

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[471] de Rooij et al. Prenatal Undernutrition and Autonomic Function in Adulthood. Psychosom Med. 2016 Nov/Dec;78(9):991-997. doi: 10.1097/PSY.0000000000000393. PMID: 27606796; PMCID: PMC5097462.

[472] Dominguez-Salas et al. Maternal nutritional status, C(1) metabolism and offspring DNA methylation: a review of current evidence in human subjects. Proc Nutr Soc. 2012 Feb;71(1):154-65. doi: 10.1017/S0029665111003338. Epub 2011 Nov 29. PMID: 22124338; PMCID: PMC3491641.

[473] Gabbianelli and Damiani. Epigenetics and neurodegeneration: role of early-life nutrition. J Nutr Biochem. 2018 Jul;57:1-13. doi: 10.1016/j.jnutbio.2018.01.014. Epub 2018 Feb 9. PMID: 29529489.

[474] Tate et al. COSMIC: the Catalogue Of Somatic Mutations In Cancer. Nucleic Acids Res. 2019 Jan 8;47(D1):D941-D947. doi: 10.1093/nar/gky1015. PMID: 30371878; PMCID: PMC6323903.

[475] Islam and Alexandrov LB. Bioinformatic Methods to Identify Mutational Signatures in Cancer. Methods Mol Biol. 2021;2185:447-473. doi: 10.1007/978-1-0716-0810-4_28. PMID: 33165866.

[476] Jeremias et al. Prospects for incorporation of epigenetic biomarkers in human health and environmental risk assessment of chemicals. Biol Rev Camb Philos Soc. 2020 Jun;95(3):822-846. doi: 10.1111/brv.12589. Epub 2020 Feb 11. PMID: 32045110.

[477] Nurk et al. The complete sequence of a human genome. Science. 2022 Apr;376(6588):44-53. doi: 10.1126/science.abj6987. Epub 2022 Mar 31. PMID: 35357919; PMCID: PMC9186530.

[478] Gershman et al. Epigenetic patterns in a complete human genome. Science. 2022 Apr;376(6588):eabj5089. doi: 10.1126/science.abj5089. Epub 2022 Apr 1. PMID: 35357915; PMCID: PMC9170183.

[479] LaSalle JM. Epigenomic signatures reveal mechanistic clues and predictive markers for autism spectrum disorder. Mol Psychiatry. 2023 Jan 17. doi: 10.1038/s41380-022-01917-9. Epub ahead of print. PMID: 36650278.

brain gene transcription, transcription factor binding, and chromatin. For example, the discovery of NHIP (neuronal hypoxia inducible, placenta associated) through an epigenome-wide association in placenta identified a common genetic risk for ASD that was modified by prenatal vitamin use.

While epigenomic signatures are distinct between different genetic syndromic causes of ASD, bivalent chromatin and some convergent gene pathways are consistently epigenetically altered in both syndromic and idiopathic ASD, as well as some environmental exposures. Together, these epigenomic signatures hold promising clues towards improved early prediction and prevention of ASD as well as genes and gene pathways to target for pharmacological interventions. The author suggests that future advancements in single cell and multi-omic technologies, machine learning, as well as non-invasive screening of epigenomic signatures during pregnancy or newborn periods are expected to continue to impact the translatability of the recent discoveries in epigenomics to precision public health.

The study by Schmidt et al., examined pregnancy and environmental factors collected by maternal interviews as predictors of placental DNA methylation, including partially methylated domains (PMDs), an embryonic feature of the placental methylome.[480] The study analyzed DNA methylation data from MethylC-seq analysis of 47 placentas of children clinically diagnosed at 3 years with autism spectrum disorder (ASD) or typical development using standardized assessments. The data was examined in relation to various factors, including child's gestational age, birthweight, and diagnosis; maternal pre-pregnancy body mass index, smoking, education, parity, height, prenatal vitamin and folate intake; home ownership; pesticides professionally applied to lawns or gardens or inside homes, pet flea/tick pouches, collars, or soaps/shampoos used in the 3 months prior to or during pregnancy. The strongest and most robust associations were found between pesticides professionally applied outside the home and higher average methylation over PMDs and a reduced proportion of the genome in PMDs. The authors suggest that pesticide exposures could alter placental DNA methylation more than other factors.

The study by Dou et al examined the use of prenatal vitamins in the first month of pregnancy in relation to cord blood and placenta DNA methylation in two prospective pregnancy cohorts: the Early Autism Risk Longitudinal Investigation (EARLI) and Markers of Autism Risk Learning Early Signs (MARBLES) studies.[481] In the placenta, prenatal vitamin intake was marginally associated with lower mean array-wide DNA methylation in EARLI, and associated with lower mean array-wide DNA methylation in MARBLES. There was little consistency in the associations between prenatal vitamin intake and single DNA methylation site effect estimates across cohorts and tissues, with only a few overlapping sites with correlated effect estimates. However, the single DNA methylation sites with p-value < 0.01 were consistently enriched in neuronal developmental pathways. The authors conclude that their findings suggest that prenatal vitamin intake in the first month of pregnancy may be related to lower placental global DNA methylation and related to DNA methylation in brain-related pathways in both placenta and cord blood.

[480] Schmidt et al. Self-reported pregnancy exposures and placental DNA methylation in the MARBLES prospective autism sibling study. Environ Epigenet. 2016 Dec;2(4):dvw024. doi: 10.1093/eep/dvw024. Epub 2016 Dec 1. PMID: 28781890; PMCID: PMC5538262.

[481] Dou et al. Prenatal vitamin intake in first month of pregnancy and DNA methylation in cord blood and placenta in two prospective cohorts. Epigenetics Chromatin. 2022 Aug 2;15(1):28. doi: 10.1186/s13072-022-00460-9. PMID: 35918756; PMCID: PMC9344645.

The study by Mordaunt reported differences in cord blood DNA methylation between children later diagnosed with autism spectrum disorder (ASD) and typically developing controls.[482] The study analyzed DNA methylation data from cord blood samples of 152 children clinically diagnosed at 3 years with ASD or typical development using standardized assessments. The data was examined in relation to numerous factors, including child's gestational age, birthweight, and diagnosis. At birth, prior to the diagnosis of ASD, a distinct DNA methylation signature was detected in cord blood over regulatory regions and genes relevant to early fetal neurodevelopment. Differential cord methylation in ASD supports the developmental and sex-biased etiology of ASD and provides novel insights into potential mechanisms underlying the disorder.

The article by Schroeder et al. discusses the use of placental methylome analysis as a potential tool for predictive ASD biomarkers in high-risk families.[483] The study used data from the MARBLES (Markers of Autism Risk in Babies: Learning Early Signs) prospective study and applied whole genome bisulfite sequencing to investigate human term placentas. The study found that while human placental methylomes have highly reproducible PMD and HMD locations, there is a greater variation between individuals in methylation levels over PMDs than HMDs due to both sampling and individual variability. In a comparison of methylation differences in placental samples from 24 ASD and 23 typically developing (TD) children, a HMD containing a putative fetal brain enhancer near DLL1 was found to reach genome-wide significance and was validated for significantly higher methylation in ASD by pyrosequencing. The study concludes that their results suggest that the placenta could be an informative surrogate tissue for predictive ASD biomarkers in high-risk families.

Convergent Pathways in ASD

The study examined the transcriptional differences between iPSC-derived cortical neurons from patients with idiopathic ASD and unaffected controls over a 135-day course of neuronal differentiation.[484] The study found that ASD-specific misregulation of genes was involved in neuronal differentiation, axon guidance, cell migration, DNA and RNA metabolism, and neural region patterning. Furthermore, functional analysis revealed defects in neuronal migration and electrophysiological activity, providing compelling support for the transcriptome analysis data. This study identified common processes altered in early neuronal development and corticogenesis that may contribute to ASD pathogenesis.

A similar study used organoid models of the human cerebral cortex to identify cell-type-specific developmental abnormalities that result from haploinsufficiency in three ASD risk genes—SUV420H1 (also known as KMT5B), ARID1B and CHD8—in multiple cell lines from different donors.[485] The authors reported that each of the three mutations confers asynchronous development of two main cortical neuronal lineages γ-aminobutyric-acid-releasing (GABAergic) neurons and deep-layer excitatory projection neurons but acts through largely distinct molecular pathways. Although these phenotypes are

[482] Mordaunt et al. Cord blood DNA methylome in newborns later diagnosed with autism spectrum disorder reflects early dysregulation of neurodevelopmental and X-linked genes. Genome Med. 2020 Oct 14;12(1):88. doi: 10.1186/s13073-020-00785-8. PMID: 33054850; PMCID: PMC7559201.

[483] Schroeder et al. Placental methylome analysis from a prospective autism study. Mol Autism. 2016 Dec 15;7:51. doi: 10.1186/s13229-016-0114-8. PMID: 28018572; PMCID: PMC5159983.

[484] DeRosa et al. Convergent Pathways in Idiopathic Autism Revealed by Time Course Transcriptomic Analysis of Patient-Derived Neurons. Sci Rep 8, 8423 (2018). https://doi.org/10.1038/s41598-018-26495-1

[485] Paulsen, B., Velasco, S., Kedaigle, A.J. et al. Autism genes converge on asynchronous development of shared neuron classes. Nature 602, 268–273 (2022). https://doi.org/10.1038/s41586-021-04358-6

consistent across cell lines, their expressivity is influenced by the individual genomic context, in a manner that is dependent on both the risk gene and the developmental defect. This study identified cell-type-specific neurodevelopmental abnormalities that are shared across ASD risk genes and are finely modulated by human genomic context, finding convergence in the neurobiological basis of how different risk genes contribute to ASD pathology.

EMBRYOLOGY AND TERATOLOGY

The science of embryology has a long and well-developed knowledge base pertaining to human gestation and fetal development. It also provides tools to scientists addressing questions of causation. As such, this discipline represents an important resource and discipline available to scientists addressing questions of causation.

For instance, embryologists have long since worked out the sequence and timing for the development of specific organs and organ systems in most species. Using this data and information, it is possible to know during which week of gestation a particular organ or system developed, and at what time during gestation its development has been completed. In broad terms, damage to organ systems caused by teratogenic or *in utero* insults can be often linked to specific gestational periods, termed critical windows of development (Figure 34). Of course, if no toxic insult occurred at this time that would argue against a chemical etiology for those malformations. On the other hand, where the time of the insult and the damaged organs match up, that can be important support for environmental causation.

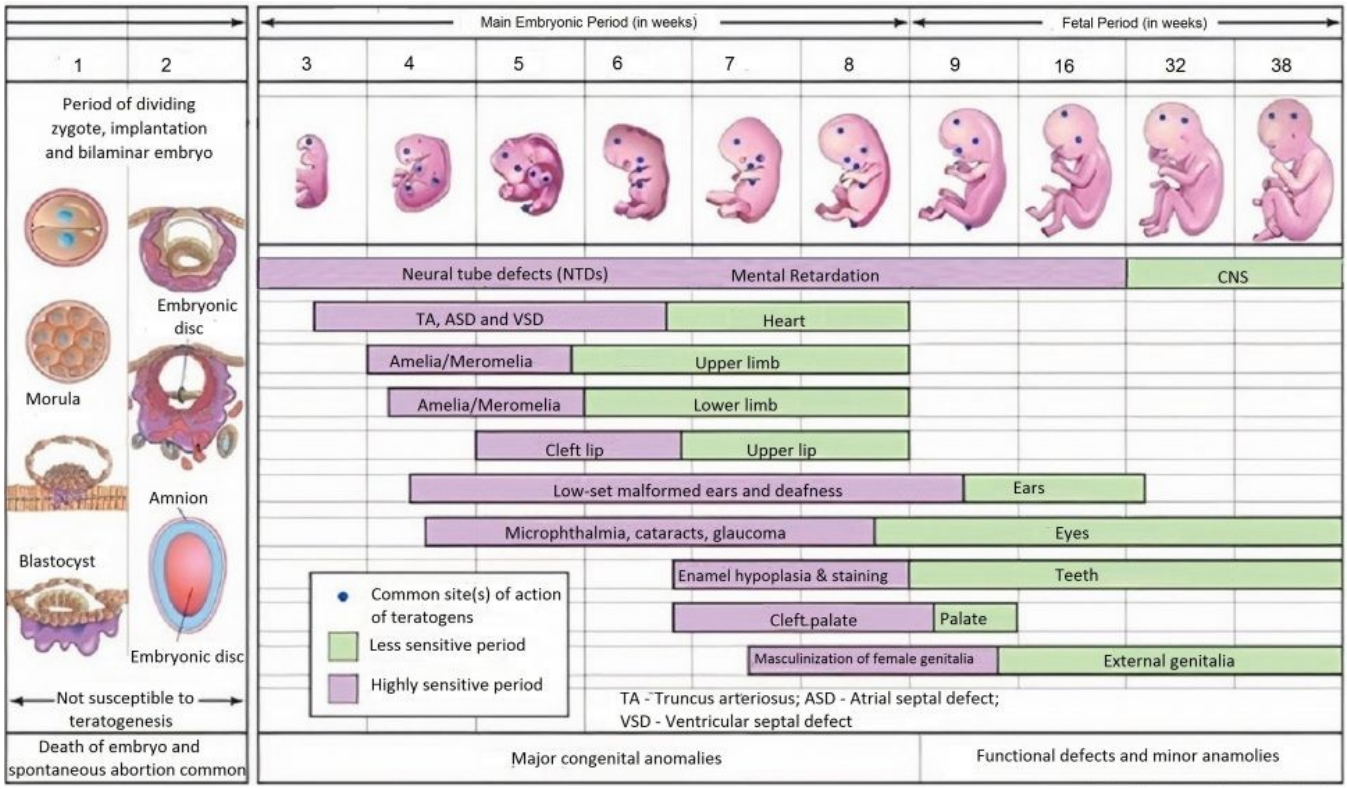


Figure 34. Critical Windows of Development. Depending on the nature of the toxic exposure, death or congenital malformations may result during the embryonic period. During the fetal period, the majority

of organ development is complete, so functional defects or minor anomalies are more likely from toxic exposures.[486]

Teratology is the branch of Embryology that focuses on environmental insults that have the capacity to damage embryological processes critical to the formation of healthy organs and systems. Teratology is the study of abnormal embryonic development. The founding principles of the modern study of teratogens were initially articulated by James G. Wilson (1973), co-founder of The Teratology Society, in his monograph *Environment and Birth Defects.*[487] These six principles remain important underpinnings of teratology, shepherding the study and knowledge of teratogens and their effects on developing organisms. These scientific principles continue to be employed when attempting to understand the potential of an environmental compound or pharmaceutical product to disrupt normal embryogenesis. These six principles are as follows:

- **Principle #1**: *Susceptibility to teratogenesis depends on the genotype of the conceptus and the manner in which this interacts with adverse environmental factors.* This principle speaks to the fact that there are genetically determined differences in susceptibility between individuals, with some being more sensitive to the teratogenic effects of a compound than others.

- **Principle #2**: *Susceptibility to teratogenesis varies with the developmental stage at the time of exposure to an adverse influence.* There are critical periods of susceptibility to agents and organ systems affected by these agents. This means that you cannot expect to induce cleft palate, for example, in a fetus if the exposure to the drug/compound takes place after the palate has formed. This principle applies to all of the major organs and structures of a developing embryo.

- **Principle #3**: *Teratogenic agents act in specific ways on developing cells and tissues to initiate sequences of abnormal developmental events.* This means that the compound disrupts normal development by interfering with specific physiological requirements of a developing organism. Teratogens act via specific mechanisms to cause birth defects. Unfortunately, we do not always know what these teratogenic mechanisms of action are, so that we cannot always develop effective interventions.

- **Principle #4**: *The access of adverse influences to developing tissues depends on the nature of the influence.* This means that there are several different factors that affect the ability of a teratogen to physically interact with a developing conceptus, such as the nature of the agent itself, route and degree of maternal exposure, rate of placental transfer and systemic absorption, and nature of the maternal and embryonic/fetal genotypes. Simply put, there are different exposure routes depending on whether the agent is a chemical, a virus, an X-ray or microwave, which influences the method by which the substance in question enters the mother's body to gain access to the developing embryo.

- **Principle #5**: *There are four manifestations of deviant development (Death, Malformation, Growth Retardation and Functional Defect).* This means that a compound could be considered to be a teratogen even if it did not cause structural malformations in an embryo. A substance that

[486] From Moore and Persaud. The Developing Human. 10th edition. Elsevier Health Sciences. ISBN: 9780323313483
[487] Wilson, James Grave. Environment and Birth Defects. New York: Academic Press, 1973.

causes the embryo to die, for whatever reasons, is a teratogen. Same is true for compounds that cause growth restrictions or adversely impacts mental development, even in the absence of structural malformations.

- **Principle #6**: *Manifestations of deviant development increase in frequency and degree as dosage increases from the No Observable Adverse Effect Level (NOAEL) to* a *dose producing 100% Lethality (LD100).* This means that as you increase the concentration of the drug exposure, you might expect to find a higher prevalence of malformed infants and that the severity of the malformations might also be expected to increase in severity with increasing dosage.

The field of teratology captured the public's attention in the early 1960s, when the morning sickness medication, Thalidomide, was determined to be the cause of thousands of children being born with limb reductions, known as phocomelia, as well as other congenital malformations.[488] This may have been the most significant event in the development and implementation of drug testing regulations that are intended to protect expectant women and their unborn children from the teratogenicity of clinically available medications.

Teratologists are typically well-trained embryologists who readily appreciate the fact that a causation analysis cannot be limited to, or founded upon, the absence of data respecting a particular unusual or rare outcome. From an embryological perspective, numbers of rare outcomes or the presence of multiple anomalies are usually compelling evidence for an environmental etiology. Finally, and of particular importance, embryological studies have clearly established that the fetus is far more vulnerable and thus far more susceptible to toxic insult and injury than are adult human beings. Accordingly, in assessing exposure levels, it must be appreciated that levels thought to be safe for adult exposures, will not necessarily be safe to the developing fetus.

This important methodological insight flows from the fact that, especially in the early weeks of gestation, many different organs and organ systems are being created simultaneously in highly interdependent and dynamic processes. Gestation begins with a single undifferentiated cell and over time these cells begin to differentiate as the fetus evolves from a cluster of identical cells to an ever more complex entity with a multitude of organs, and tissues.

[488] Friedman JM. ABCDXXX: The obscenity of postmarketing surveillance for teratogenic effects. Birth Defects Res A Clin Mol Teratol. 2012 Aug;94(8):670-6. doi: 10.1002/bdra.23043. Epub 2012 Jul 11. PMID: 22786781.

RULING OUT ALTERNATIVE EXPLANATIONS

I have reviewed the report prepared by ChemRisk. Regarding background information, they included a reference to a report by the Minnesota Department of Health (MDH). It should be noted that the reference doses used in that study are based on background exposures to PAP and APAP, not on pharmaceutical exposures to APAP. As indicated in **Acetaminophen and Aniline**, the pharmaceutical intake of APAP results in an approximately 1000-fold increase in exposure levels. This report, viewed in this context of pharmaceutical dosing does not present a 1700-fold margin of safety, only a relatively small margin of safety that is approximately 1.7 to 2.4 times therapeutic pharmaceutical exposures. This is similar to the margin of safety between APAP recommended dosages (1g x 4 = 4g/day) and the potential for toxicity at intakes ≥ 150 mg/kg (~7.5 g/day in adults). In this example, doubling the recommended dose (2g x 4 = 8g), would also exceed the margin of safety for APAP and liver damage.

It is important to understand that the dose used by the MDH was based on background environmental intake, not pharmaceutical intake. I will present the MDH exposure data compared to pharmaceutical exposures. The derivations of the MDH reference doses (RfDs) ranged from 0.093 to 0.28 mg/kg/day. A recommended human dose (1000 mg), is a human reference dose of 16.7mg/Kg, based on a 60Kg human. This single dose is 180 to 60 times higher than the MDH RfD, and a recommended daily dosage, once every six hours (1g x 4) produces an APAP dosage of 66.8mg/Kg/day, and this is 720 to 240 times the RfD.

The information presented by ChemRisk looks very different with this pharmaceutical dose in mind, as ChemRisk quotes the MDH report,

> "the overall weight-of-evidence suggests that acetaminophen is not a developmental toxicant in humans" and that "[e]xperimental animal studies do not suggest increased malformations from therapeutic use of acetaminophen during pregnancy" (MDH, 2015: p. 5). Additionally, it was noted that "[n]o effects on pregnancy or offspring were reported in several laboratory animal studies at human equivalent doses up to over 500 times higher than the acute and short-term RfDs" while "a continuous breeding animal study, effects on reduced fertility and reproduction were observed at human equivalent dose 800 times higher than the acute and short-term RfDs" (MDH, 2015: p. 5). Finally, it was noted that "[a]cetaminophen is not considered to be a neurotoxicant based on lack of secondary observations in animal studies" and that "[i]n laboratory animals, clinical neurotoxicity symptoms were reported only at very high doses over 1,700 times higher than the RfDs" (MDH, 2015: p. 5).

No effects at over 500 times the RfDs, would be a good margin of safety, but this safety is relative to environmental exposures, such as municipal drinking water. This exposure is within the range of a daily pharmaceutical intake, as the daily dosage of 4g/day for APAP results in 720 to 240 times the RfD. Likewise, the continuous breeding animal study reported reduced fertility and reproduction at 800X times the acute and short-term RfDs, which is actual only 1.1-3.3 times a daily "therapeutic" intake. Much of the ChemRisk report presents the data in this fashion, which could be rather misleading without the appropriate context.

As another example of this type of presentation, the ChemRisk analysis incorrectly combine rat and mouse dosing and compare directly to human dosages. Specifically, ChemRisk compares rat and mouse dosing

to human dosages without converting to HEDs, human equivalent dosages, based on mg/m^2. The absence of this surface area conversion, which is used to account for metabolic differences between species, results in ChemRink incorrectly suggesting that dosages used in animal studies are too high to be relevant for human exposures or are overwhelmingly expected to cause hepatoxicity. The majority of mouse and rat studies are within therapeutic dosages, as indicated in Table 6 and Table 9. Dosages used in mice and rats, respectively, are reported and include experimentally determined therapeutic antinociceptive dosages (ED_{50}) for mice and rats, and estimated HEDs below the tables, reported and calculated as previously indicated, see Table 1 and Human Equivalent Dosages.

I have considered the following explanations, as presented by Alwan et al.[489] (2021) in response to Bauer et al (2021).[490] Alwan proposes, (1) "failure to account for confounding, and elements of bias that make interpretation of the data challenging", (2) "confusion with other analgesic drugs", (3) "Residual confounding from shared co-morbidities and genetic and environmental", (4) "migraine or fever", or (5) "underlying genetic susceptibility to neurodevelopmental and psychiatric conditions." Confounding was addressed, and multiple study designs addressed confounding with different approaches, yet the individual studies and meta-analyses still detected statistically significant associations. Regarding confusion, other analgesics have not reported the same risks, so such confusion should bias towards the null, not away. Regarding genes and environment, specific genetic risk factors are expected to be identified showing increased risks for individuals or populations; this is expected and consistent with Wilson's Principles. Regarding migraine or fever, other medications also pose risks during pregnancy, and these confounders were controlled for in studies individually and in meta-analyses. Regarding a familial risk or underlying genetic susceptibility, there are specific genes that can cause ASD and ADHD, but that does not negate the population burden and risk of exposing pregnant women to APAP, that increased the prevalence in that population above background. Pregnancy itself also increases the risk of APAP toxicity, and having underlying genetic risk factors is expected to increase the severity of disease or the incidence in APAP exposed population.

Bauer presents the following graphic, which summarizes animal data in "a call for precautionary action."

[489] Alwan et al. Paracetamol use in pregnancy - caution over causal inference from available data. Nat Rev Endocrinol. 2022 Mar;18(3):190. doi: 10.1038/s41574-021-00606-x. PMID: 34907341.
[490] Bauer et al. Reply to 'Paracetamol use in pregnancy - caution over causal inference from available data'; 'Handle with care - interpretation, synthesis and dissemination of data on paracetamol in pregnancy'. Nat Rev Endocrinol. 2022 Mar;18(3):192. doi: 10.1038/s41574-021-00610-1. PMID: 34907342.

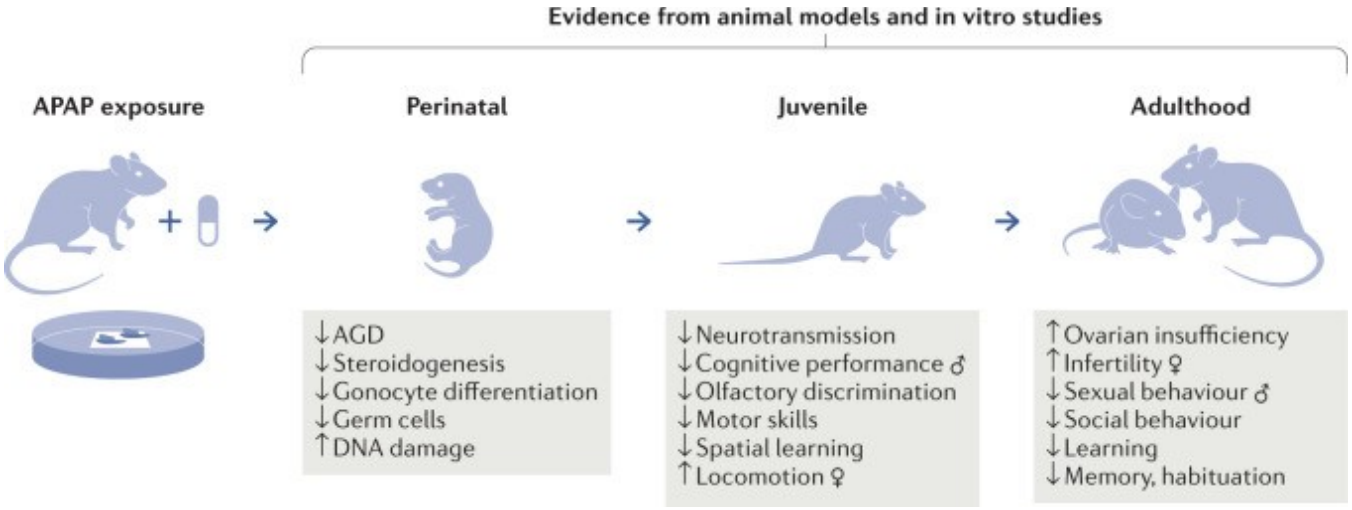


Figure 35. Evidence of Acetaminophen (APAP) Disruption of Reproductive and Neurological Development from Animal Studies (from Bauer et al. 2022).

As reviewed above, there is "**clear evidence" of reproductive, developmental, and neurodevelopmental toxicity.** The NTP reports there is "equivocal evidence" of carcinogenicity based on leukemia in female rats, which is consistent with oxidative damage and DNA damage, known to be produced by the APAP metabolite NAPQI. I find there is **"some evidence"** of carcinogenicity based on the similar cancers found in female rats by NTP (1993), and the increased incidence of leukemia in human taking APAP, as a function of dose and duration.

Regarding reproductive or developmental toxicity:

- •Clear evidence of reproductive toxicity is demonstrated by a dose-related effect on fertility or fecundity, or by changes in multiple interrelated reproductive parameters of sufficient magnitude that by weight of evidence implies a compromise in reproductive function.
- •Clear evidence of developmental toxicity is demonstrated by data that indicate a dose-related effect on one or more of its four elements (embryo-fetal death, structural malformations, growth retardation or functional deficits) that is not secondary to overt maternal toxicity.

Regarding carcinogenicity:

- •Some evidence of carcinogenic activity is demonstrated by studies that are interpreted as showing a chemical-related increased incidence of neoplasms (malignant, benign, or combined) in which the strength of the response is less than that required for clear evidence.

Bauer presents the following graphic, which summarize human data in "a call for precautionary action."

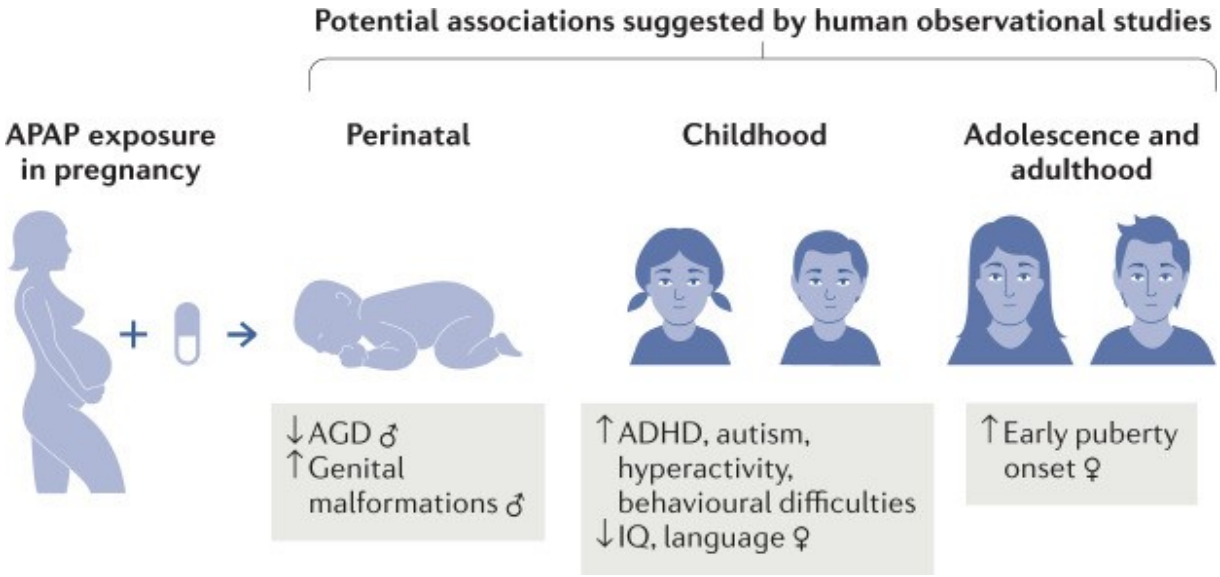


Figure 36. Associations between Prenatal APAP Exposure, Reproductive and Neurobehavioural Development Suggested from Observational Human Studies ***(From Bauer et al. 2022).***

Bauer presents the following regarding use of medications during pregnancy,

> These findings suggest that many people view APAP as conveying negligible risk, instead of being a 'true medication' with potential adverse effects.[491]

Much like the evidence initially rejected by doctors that smoking could cause lung cancer, the historic usage of APAP has built a false belief of safety. Regulatory and research toxicity studies, based on both clinical and animal testing, indicate an increase in risk with frequent or continuous APAP usage. This false perception of safety is demonstrated by APAP being considered safe, while covertly being the primary cause of acute liver failure in the United States. While historic usage creates claims of safety to an unknowing public, as did smoking, these false beliefs must yield to factual evidence and scientific data. The evidence is clear, APAP taken during pregnancy, at therapeutic dosages, is sufficient to cause and increase the incidence of, as a function of duration and timing, reproductive, developmental, and neurodevelopmental toxicity.

[491] Bauer et al. Reply to 'Paracetamol use in pregnancy - caution over causal inference from available data'; 'Handle with care - interpretation, synthesis and dissemination of data on paracetamol in pregnancy'. Nat Rev Endocrinol. 2022 Mar;18(3):192. doi: 10.1038/s41574-021-00610-1. PMID: 34907342.

THE CAUSAL RELATIONSHIP BETWEEN ACETAMINOPHEN AND NEURODEVELOPENTAL TOXICITY

A systematic review of the genetic, reproductive, developmental, and neurodevelopmental toxicity literature indicates that APAP and metabolites thereof are associated with reproductive damage, developmental toxicity, teratogenicity, neurodevelopmental toxicity, and neurobehavioral and neurodevelopmental delays. Association, however, does not alone prove causation and further analysis is necessary. Sir Bradford Hill proposed a set of criteria to determine causation between exposure and outcome. These criteria are accepted in the scientific community as a reliable and viable method of determining the potential existence of causality. Thus, I next review each of the Bradford Hill criteria in terms of their importance for establishing that there is a causal relationship between acetaminophen and neurodevelopmental toxicity.

1. Strength of Association:

   The greater the magnitude of the association between an environmental exposure and an adverse outcome, the more likely it is that the exposure caused the outcome. In general, an odds ratio between 1 and 2 is deemed low, a ratio from 2 to 6 is deemed moderate, and a ratio above 6 is deemed high. A low strength of association, however, is not by itself sufficient to reject a cause-and-effect hypothesis. As Hill stated: "We must not be too ready to dismiss a cause-and-effect hypothesis merely on the grounds that the observed association appears to be slight," because "[t]here are many occasions in medicine when this is in truth so."[492] We must carefully assess whether other factors (confounders) may be responsible for an observed low or more moderate strength association. And if high quality studies reasonably account for identified confounders and still show an association (particularly a dose-response association), then the mere potential that some other unidentified factor is responsible for the excess risk is not a basis for rejecting the causation hypothesis. As Hill explained, "If we cannot detect it or reasonably infer a specific [alternative cause], then in such circumstances I think we are reasonably entitled to reject the vague contention of the armchair critic 'you can't prove it, there may be such a feature'."

   Numerous studies are cited that report an increased risk for neurodevelopmental toxicity, with ADHD and ASD as common endpoints, and APAP exposure. The magnitude of this risk is low in the majority of studies (1<OR<2), but moderate risk (OR>2) is reported with higher acetaminophen exposures. Specifically, there are reported risks of ADHD with APAP in meconium resulting in an OR 2.43 (95% CI, 1.41-4.21); with a dose-response doubling of exposure increasing the odds of ADHD by 10% (OR, 1.10; 95% CI, 1.02-1.19), and when grouped based on high levels of APAP detected in meconium, the odds of ADHD were more than 4-fold, OR 4.10 (95% CI, 2.41-6.95).[493] Measurements of unmetabolized APAP in umbilical cord blood at >50th percentile were also associated with higher odds of ADHD diagnosis (aOR: 2.10, 95% CI, 1.43- 3.11, $p < 0.001$).[494] While fever is proposed as a confounder of these reported associations, termed confounding by

[492] Hill, A.B. The environment and disease: association or causation? (1965) Proc R Soc Med. 1965 May;58(5):295-300. PMID: 14283879; PMCID: PMC1898525.

[493] Baker et al. Association of Prenatal Acetaminophen Exposure Measured in Meconium With Adverse Birth Outcomes in a Canadian Birth Cohort. Front Pediatr. 2022 Apr 5;10:828089. doi: 10.3389/fped.2022.828089. PMID: 35450103; PMCID: PMC9017809.

[494] Anand et al. Perinatal Acetaminophen Exposure and Childhood Attention-Deficit/Hyperactivity Disorder (ADHD): Exploring the Role of Umbilical Cord Plasma Metabolites in Oxidative Stress Pathways. Brain Sci. 2021 Sep 30;11(10):1302. doi: 10.3390/brainsci11101302. PMID: 34679367; PMCID: PMC8533963.

indication, there is a lack of evidence to support that other medications used for the same indications (fever), such as ibuprofen or aspirin, are associated with ADHD or ASD risks, which means that the presence of fever does not explain the increased risk associated with exposure to APAP. In addition, there are factors that bias studies towards the null, which makes the association weaker. Overall, meta-analyses report statistically significant associations between acetaminophen exposure and ASD[495] and ADHD[496] risk. While some individual studies have not supported specific associations, the totality of data is consistent with "clear evidence of developmental toxicity" as stated by the National Toxicology Program (NTP), "Clear evidence of developmental toxicity is demonstrated by data that indicate a dose-related effect[497] on one or more of its four elements (embryo-fetal death, structural malformations, growth retardation or functional deficits) that is not secondary to overt maternal toxicity."

Moreover, animal studies remove indications and other confounders, and as supported by numerous well controlled animal studies, APAP has been shown to cause neurodevelopmental toxicity and impaired learning and social behaviors consistent with ASD and ADHD at clinically relevant therapeutic dosages. These reports meet the criterion of strength of association.

2. Consistency of the Evidence:
This criterion assesses whether the observed association has been "repeatedly observed by different persons, in different places, circumstances, and times," which then allows us to "justifiably infer that the association is not due to some constant error or fallacy that permeates every inquiry." [498]

The association between fetal exposure to APAP and ASD/ADHD has been reported in epidemiological studies performed by several different laboratories working independently and publishing in different peer-reviewed journals. The association has been observed in Spanish, Brazilian, Swedish, Norwegian, English, and Danish cohorts, each independently reporting neurodevelopmental impacts, including increased ASD, ADHD, and associated behaviors such as language delays.

The epidemiological studies have also used differing techniques and methodologies, including prospective cohort studies, followed longitudinally without foreknowledge of the outcomes.

The meta-analyses combine data from multiple cohort studies, indicating a consistent pattern of association between acetaminophen exposure and ASD and ADHD risk across different populations. While some studies have not supported specific associations, the totality of data is consistent with "Clear evidence of developmental toxicity." As stated by the National Toxicology Program (NTP), "Clear evidence of developmental toxicity is demonstrated by data that indicate a

[495] Alemany et al. Prenatal and postnatal exposure to acetaminophen in relation to autism spectrum and attention-deficit and hyperactivity symptoms in childhood: Meta-analysis in six European population-based cohorts. Eur J Epidemiol. 2021 Oct;36(10):993-1004. doi: 10.1007/s10654-021-00754-4. Epub 2021 May 28. PMID: 34046850; PMCID: PMC8542535.
[496] Ricci et al. In utero acetaminophen exposure and child neurodevelopmental outcomes: Systematic review and meta-analysis. Paediatr Perinat Epidemiol. 2023 Mar 20. doi: 10.1111/ppe.12963. Epub ahead of print. PMID: 36939050.
[497] The term "dose-related" describes any dose-response relationship, recognizing that the test article-related responses for some endpoints may be non-monotonic due to saturation of exposure or effect, overlapping dose-response behaviors, changes in immunologic manifestations at different dose levels or other phenomena.
[498] Hill, A.B. The environment and disease: association or causation? (1965) Proc R Soc Med. 1965 May;58(5):295-300. PMID: 14283879; PMCID: PMC1898525.

dose-related effect[499] on one or more of its four elements (embryo-fetal death, structural malformations, growth retardation or functional deficits) that is not secondary to overt maternal toxicity." In addition, well-controlled animal studies from at least [X] different laboratories have independently demonstrated "clear evidence" that perinatal APAP exposure to mice and rats, at doses equivalent to human therapeutic dosages, caused neurodevelopmental toxicity, changes in the brain, and impaired learning and socialization. The consistency criterion is met.

3. Specificity:
A high degree of specificity exists to the extent exposure to one factor (and no other) causes one disease (and no other). As a practical matter, high specificity can be detected when a rare environmental event is associated with a rare disease. The usage of APAP is common to a majority (>50%) of human pregnancies and the frequency of ASD (~2.2%) and ADHD (~10%) are increasing in the USA. The association between APAP exposure and ASD or ADHD is not specific since APAP exposure is common and can cause other toxicities, including hepatotoxicity. ASD and ADHD can also be caused by other exposures, such as *in utero* valproic acid or mercury exposure. When controlled for in animal studies or adjusted for in epidemiological studies, specific interactions are present. The specificity criterion is not fully met.

4. Temporality:
Epidemiological and animal studies demonstrated that exposure preceded outcome. Studies have shown that the effect of APAP is dependent on the timing of exposure in relation to specific developmental processes. The exposure to acetaminophen (during pregnancy) precedes the outcome (development of ASD or ADHD) temporally, supporting a temporal relationship. The temporality criterion is met.

5. Biologic Gradient (dose-response):
The meta-regression analyses indicate that the association between APAP exposure and ASD risk increased with the child's age at follow-up and the mean duration of exposure. This supports a biological gradient, as a longer duration of exposure and older age were associated with a higher risk of ADHD. The biological gradient criterion is met for ASD.

Meta-analyses did not explicitly report a dose-response relationship between APAP exposure levels and the risk of ADHD. Two studies looked at APAP in meconium, one supporting a dose-response interaction between APAP and ADHD. Another study looked at cord blood and supported a dose-response interaction. The biological gradient criterion is supported for ADHD.

Dose-response is also reported throughout testing for reproductive and developmental toxicity. APAP and the metabolite NAPQI increase oxidative damage in a dose dependent manner, which in turn produces genetic, reproductive, developmental, and neurodevelopmental toxicity. The biological gradient criterion for neurodevelopmental toxicity is supported.

6. Biologic Plausibility:
There is biological plausibility for the interaction, as APAP can cross the placental barrier and affect

[499] The term "dose-related" describes any dose-response relationship, recognizing that the test article-related responses for some endpoints may be non-monotonic due to saturation of exposure or effect, overlapping dose-response behaviors, changes in immunologic manifestations at different dose levels, or other phenomena.

fetal neurodevelopment. The published adverse outcome pathway (OECD 20) also supports a causal-plausible interaction between APAP and oxidative stress and neurodevelopmental adverse outcomes. The brain is particularly vulnerable to oxidative stress. When GSH is depleted, free radicals—including NAPQI and other endogenous radicals that normally rely on GSH buffering—cause damage that disrupts normal metabolic, cellular, and neurodevelopment of brain tissues in multiple different ways. NAPQI can bind molecules within cells and damage or disrupt the normal functions of proteins, lipids, DNA, and other components of the cell. Oxidative stress induced damage disrupts the differentiation of stem cells into neurons, proliferation of cells, migration of neural cells, and mitochondrial functioning, and can cause overt toxicity and cell death.

In addition to generating oxidative stress, APAP operates on a parallel mechanism by disrupting endocannabinoid, prostaglandin, and serotonergic signaling. The enzyme FAAH, which produces the APAP metabolite AM404, is expressed in neurons and throughout the central nervous system. APAP and its metabolite AM404 disrupt the endocannabinoid system by increasing levels of anandamide, the primary CB1 agonist, and by activating both CB1 and CB2 receptors. APAP also inhibits synthesis of prostaglandins. The effect of APAP on the endocannabinoid system and prostaglandin synthesis is sufficient to relieve pain and fever in an adult. But the normal operation of the endocannabinoid system and prostaglandins are essential for normal brain development. Endocannabinoids regulate the number and division rate of stem cells that differentiate into neural cells, the type of neuronal cell (neuron or glia) produced from neural stem cells, axon growth, and axon positioning. Prostaglandins are used to signal the formation of dendritic spines, and to influence arborization and pruning of connections. AM404 is also a potent activator of TRPV1, which can activate an inflammatory response in the brain. APAP also has indirect effects on serotonergic signaling, and such signaling facilitates neurogenesis, cell migration, synaptogenesis, and synaptic plasticity. The neurodevelopmental impact of acetaminophen on ADHD and ASD risk are biologically plausible and supported.

7. Coherence:

This criterion assesses whether the cause-and effect interpretation of the data is consistent with or would "seriously conflict with the generally known facts of the natural history and biology of the disease."[500] Hill identifies as examples whether causation is consistent with any sex differences in the disease, and whether we can isolate the particular factor in the environmental exposure that can cause the outcome in a laboratory setting (e.g. a chemical in cigarette smoke that causes cancer on the skin of laboratory animals). This is fulfilled by the epidemiological, global adverse reporting, and animal studies on pre-, peri- and post-natal APAP exposures.

A causal relationship between APAP and neurodevelopmental harm is consistent with the expected timing and results of exposure. For APAP to cause neurodevelopmental harm, we would expect that exposure during key developmental windows would produce harm. Animal studies demonstrate that exposure to APAP at therapeutic doses during the Brain Growth Spurt (PD3 or PD10, but not on PD19) results in later-life changes in the brain and impaired learning and socialization. If APAP exposure at therapeutic doses can generate sufficient oxidative stress to result in neurodevelopmental toxicity, then we expect that it would also cause parallel damage on

[500] Hill, A.B. The environment and disease: association or causation? (1965) Proc R Soc Med. 1965 May;58(5):295-300. PMID: 14283879; PMCID: PMC1898525.

reproductive development and DNA oxidation damage. Human and animal studies have demonstrated that perinatal exposure to APAP is associated with reproductive toxicity and DNA oxidative damage. While adverse outcomes are seen across numerous body systems, they are linked by oxidative damage, consistent with the published APAP AOP (see, Figure 11. Oxidative Stress and Developmental Impairment of Learning and Memory).

APAP as a causative agent is consistent with sex-linked differences in ASD and ADHD. The male to female ratio in ASD has been recently estimated as 4:1.[501] The estimates of the male to female ratio for ADHD range from 3:1 to 10:1.[502] The degree of symptoms is also reported to vary between males and females. For example, males with ASD have been reported to exhibit more repetitive behaviors than females, and males with ADHD are reported to greater inattention, hyperactivity/impulsivity, and greater total ADHD symptoms than females with ADHD.[503] Consistent with these outcomes, exposure to APAP in animal studies has resulted in sex-linked differences. The association between APAP exposure and ASD and ADHD is coherent with the existing knowledge of oxidative stress and potential for hepatotoxicity and neurotoxicity with APAP exposures. The coherence criterion is met.

8. Experimental Evidence:
Randomized controlled trials for developmental toxicity, teratogenicity, or neurodevelopmental toxicity are not conducted in pregnant women for ethical reasons. The available experimental evidence from animal models consistently demonstrates dose-responsive reproductive, developmental, and neurodevelopmental toxicity with pre-, peri- and post-natal APAP exposures. This criterion is supported with available experimental evidence.

9. Analogy:
The presence of an analogous drug-disease causal relationship supports a causal inference. There are analogies with other substances that are known to have neurodevelopmentally toxic effects during pregnancy, including Δ9-THC, mercury, and valproic acid, supporting a common oxidative stress mechanism for mercury, like APAP, and chemical-pharmaceutical causes of ADHD and ASD.

I reviewed above the evidence showing that Δ9-THC from cannabis has a common mode of action with the APAP metabolite AM404. As reviewed, AM404 inhibits the uptake of anandamide, and Δ9-THC and anandamide can both act as agonists for CB1 and CB2 receptors, meaning they can bind to and activate these receptors. Altering cannabinoid signaling during development produces a shared outcome, neurodevelopmental toxicity, including ASD and ADHD.

Another analogy is mercury. As explained above, the OECD prepared an Adverse Outcome Pathway analysis on a set of chemicals that bind to sulfhydryl (thiol)-group proteins. The results

[501] Centers for Disease Control and Prevention (CDC) Prevalence of autism spectrum disorder among children aged 8 years—autism and developmental disabilities monitoring network, 11 Sites-United States, 2014 (2018, April 27). Retrieved from: https://www.cdc.gov/mmwr/volumes/67/ss/ss6706a1.htm. 10.15585/mmwr.mm6745a7

[502] Biederman J, Mick E, Faraone SV, Braaten E, Doyle A, Spencer T, et al. Influence of gender on attention deficit hyperactivity disorder in children referred to a psychiatric clinic. *Am J Psychiatr* (2002) 159:36–42. 10.1176/appi.ajp.159.1.36

[503] Mahendiran, T. et al., Sex Differences in Social Adaptive Function in Autism Spectrum Disorder and Attention-Deficit Hyperactivity Disorder (2019) Front. Psychiatry 10:607, DOI: 10.3389/ fpsyt.2019.00607

were published in December 2022 as AOP 20. Both mercury and APAP are reported to cause oxidative stress. The analysis concluded that exposure to mercury causes "oxidative stress" sufficient to "cause cellular injury and death" that disrupts "the establishment of neuronal connections and networks" and can "lead to functional impairment in learning and memory." In addition, two studies found that children living in close proximity to industrial power plant sources of mercury had significantly higher prevalence of autism.[504], [505] It has also been reported that children with autism exposed to mercury showed significantly decreased level of GSH, and exposure to mercury has been also reported in some cases of autism.[506] Oxidative damage during development produces a shared outcome, neurodevelopmental toxicity, including ASD and ADHD. Meta-analyses found a significant association between higher mercury levels in various tissues (blood, RBC, and brain) and ASD.[507] This suggests a positive correlation between mercury exposure and ASD risk, meeting the criterion of strength of association. The association between mercury exposure during pregnancy and ASD is not specific, as mercury exposure has also been associated with other adverse health effects and neurodevelopmental disorders. Thus, the specificity criterion is not fully met. Available meta-analyses do not provide detailed information on the timing of mercury exposure during pregnancy and the subsequent development of ASD in offspring. The temporality criterion cannot be fully evaluated based on the available information. The meta-analyses do not explicitly report a dose-response relationship between mercury exposure levels and the risk of ASD. Further information on the dose-response relationship is necessary to assess the biological gradient criterion. The plausibility of a causal relationship between mercury exposure during pregnancy and ASD is supported by the known neurotoxic properties of mercury. However, additional research is needed to elucidate the specific mechanisms through which mercury may contribute to the development of ASD. The association between mercury exposure during pregnancy and the development of ASD is coherent with the known neurotoxic effects of mercury. However, further coherence with other scientific knowledge and research is required for a more comprehensive assessment. Controlled experiments involving deliberate mercury exposure during pregnancy are not feasible due to ethical considerations. Therefore, the available evidence relies primarily on animal studies and observational studies, including case-control designs, as seen in meta-analyses. Based on the information provided in meta-analyses and the application of the Bradford Hill Criteria, there is evidence supporting an analogous causal relationship between mercury exposure during pregnancy and ASD.

Another analogous causal relationship is found in valproic acid. Several studies have demonstrated an association between valproic acid exposure during pregnancy and an increased risk of ASD. Valproic acid exposure during pregnancy has been linked to an increased risk of various neurodevelopmental disorders, including ASD. Although not specific to ASD alone, the fact that

[504] Windham GC, Zhang L, Gunier R, Croen LA, Grether JK: Autism spectrum disorders in relation to distribution of hazardous air pollutants in the San Francisco bay area (2006) Environ Health Perspect 2006; 114: 1438–1444.
[505] Palmer RF, Blanchard S, Wood R: Proximity to point sources of environmental mercury release as a predictor of autism prevalence (2009) Health Place; 15: 18–24.
[506] Mutter J, Naumann J, Schneider R, Walach H, Haley B: Mercury and autism: accelerating evidence? (2005) Neuro Endocrinol Lett 26: 439–446.
[507] Jafari et al. The association between mercury levels and autism spectrum disorders: A systematic review and meta-analysis. J Trace Elem Med Biol. 2017 Dec;44:289-297. doi: 10.1016/j.jtemb.2017.09.002. Epub 2017 Sep 4. PMID: 28965590.; Sulaiman et al. Exposure to Aluminum, Cadmium, and Mercury and Autism Spectrum Disorder in Children: A Systematic Review and Meta-Analysis. Chem Res Toxicol. 2020 Nov 16;33(11):2699-2718. doi: 10.1021/acs.chemrestox.0c00167. Epub 2020 Oct 12. Erratum in: Chem Res Toxicol. 2021 Jun 21;34(6):1693. PMID: 32990432.

valproic acid has been consistently associated with neurodevelopmental effects strengthens the case for a potential causal relationship. The exposure to valproic acid occurs during pregnancy, and the subsequent diagnosis of ASD in the offspring is assessed later in life. This temporal sequence supports a potential causal relationship between the exposure and the outcome. Evidence suggests that there might be a dose-response relationship between the level of valproic acid exposure during pregnancy and the risk of ASD. Higher levels of exposure or longer duration of exposure may be associated with a greater risk of ASD. Valproic acid is known to affect neurodevelopment and has been associated with various adverse effects on the developing brain. Animal studies and in vitro research support the plausibility of valproic acid's potential role in the development of ASD. The association between valproic acid exposure during pregnancy and an increased risk of ASD is coherent with our current understanding of the neurodevelopmental effects of valproic acid and the etiology of ASD. As an analogy, there is also substantial evidence suggesting a causal relationship between valproic acid exposure during pregnancy and an increased risk of ASD.

The FDA-approved label for Depakene states, "Although the available studies have methodological limitations, the weight of the evidence supports a causal association between valproate exposure in utero and subsequent adverse effects on neurodevelopment, including increases in autism spectrum disorders and attention deficit/hyperactivity disorder (ADHD)."[508]

I find that APAP carries a number of risks, including those that manifest as the primary cause of acute liver failure in the United States. There is risk of encephalopathy, both proximal (rare) and secondary to hepatic damage, with a relatively limited margin (< 2X MRHD) of safety. There are also reproductive, developmental, and neurodevelopmental risks, as presented in this structured review and report, as supported by "clear evidence" in animal studies and "clear evidence" in available human meta-analyses examining the risk of *in utero* APAP exposures.

[508] New Drug Application (NDA): 018081, Company: ABBVIE, 05/19/2020, SUPPL-71, Labeling-Package Insert, DEPAKENE, VALPROIC ACID, https://www.accessdata.fda.gov/drugsatfda_docs/label/2020/018081s071,018082s054lbl.pdf

CONCLUSIONS

Within a reasonable degree of scientific certainty, the undersigned opines:

1. A therapeutic dose of Acetaminophen (APAP) produces >1000X increase in APAP exposures over environmental background exposures to APAP (from environmental aniline).

2. Acetaminophen produces oxidative damage, reproductive, developmental, and neurodevelopmental toxicity at clinically relevant therapeutic dosages, including neurodevelopmental disorders, such as autism spectrum disorder (ASD) and attention deficit hyperactivity disorder (ADHD).

3. Therapeutic dosages of APAP taken by pregnant woman are sufficient to cause neurotoxicity, neurodevelopmental disorder, ASD, and ADHD in exposed offspring.

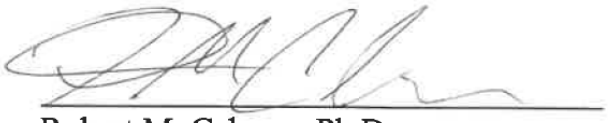
4. There exists no reasonable alternative cause or explanation for these injuries which contradict or undermine the general causation opinions expressed above.

I expressly reserve the right to amend or supplement this report and to read, review and comment upon any reports prepared by Defendants' experts.

The foregoing opinions are substantively identical to the opinions stated in my June 16, 2023, report. All opinions offered herein are held to a reasonable degree of scientific certainty.

Dated: June 22, 2023

Respectfully submitted,



Robert M. Cabrera, Ph.D.

Exhibit A

Robert M. Cabrera, Ph.D.

Baylor College of Medicine
One Baylor Plaza
Houston TX 77030

bcm.edu
[redacted]
Phone: [redacted]

Summary of Qualifications

Academic Experience

Over 15 years of experience managing an academic research laboratory
Active in pharmaceutical and environmental chemical testing for developmental toxicity
Performs nutritional research for mitigating diseases and prevention of birth defects
Lecturer for Baylor College of Medicine and Texas A&M University School of Dentistry
Writing federal grants, contracts, and scientific publications
-Awarded Multiple NIH and NIMH grants (R01 and R21) as Primary Investigator
-Authored two multi-million-dollar grants with the Environmental Protection Agency
-Authored a 12.25 million dollar contract with the Defense Threat Reduction Agency

Professional Experience

Expert witness on reproductive and developmental toxicity
Experience in review of preclinical Segment I-III studies
Greater than two years of commercial laboratory research experience
Experience with GLP and CLIA regulatory compliance
Provided scientific technical support for in-field marketing and sales

Education

09/2001-05/2006 Texas A&M University HSC-IBT Houston, TX
Ph.D. in Medical Sciences

Studied gene-environment interactions and neural development
Performed teratology studies for pharmaceuticals
Developed immunoassays for measuring autoantibody and protein-ligand interactions

09/1995 – 05/1999 Texas A&M University College Station, TX
B.A. in Biology, Minor in Chemistry

Member of Phi Eta Sigma National Honor Society.

Laboratory Experience

Supervised, coordinated, and mentored research teams
Over 20 years of experience in genetic research:
- DNA sequencing, vector design, and production of mouse models of human diseases

More than 20 years of experience conducting pharmacologic and teratogenic testing
Extensive experience in biologic assay design, fabrication, processing, and data analysis
- 15 years developing and performing immunologic and hematologic testing

Over ten years performing and managing embryonic stem cell isolation, culture, and research

Employment

10/2017 – Present **Baylor College of Medicine** **Houston, TX**
Associate Professor
Manage research studies on HIV Integrase Inhibitors.
Develop cellular and mouse models of gene-environmental interactions with birth defects.

01/2021 – Present **San Jacinto College** **Houston, TX**
Adjunct Faculty – Life Sciences - Biology
Instructor for Nutritional Sciences.

03/2021– Present **DARTox Consulting LLC** **Houston, TX**
10/2013 – 05/2020 **Teratomics Consulting LLC** **Austin, TX**
Expert witness on reproductive and developmental toxicity testing
Expert reports for multi-million dollar personal injury litigation.
Conducting research and submitting research reports to clients.

09/2016 – 05/2021 **University of Texas at Austin – University Extension** **Austin, TX**
Adjunct Faculty - Nutritional Sciences
Developed and deployed distance learning course for Nutritional Sciences.

09/2010 – 10/2017 **Dell Pediatric Research Institute** **Austin, TX**
Faculty Lecturer & Research Scientist
Manage EPA Science to Achieve Results (STAR) and Small Business Innovative Research.
Management of stem cell research group (post-doctoral, graduate, and undergraduates).
Writing grants, contracts, publications, and presenting research.

06/2006 – 09/2010 **Texas Institute for Genomic Medicine** **Houston, TX**
Research Scientist
Writing grants, contracts, publications, and scientific technical support for sales.
Conception and management for EPA and DOD funded research projects.

01/2000 – 07/2001 **Genometrix** **Woodlands, TX**
Research Associate II, Process Development
Manual and automated microarray fabrication, processing, and statistical analysis.
Design, testing, and establishing GLP protocols and quality control assays.

06/1999 – 12/1999 **SeqWright, Inc.** **Houston, TX**
Laboratory Technician
Sample processing: DNA-preps, primer design and synthesis, PCR and DNA sequencing.
Sequencing analysis, genetic assembly (Sequencher) and data distribution.

03/1995 – 05/1999 **Texas A&M University** **College Station, TX**
Laboratory Assistant
Development and testing mouse models. Phenotypic analysis of congenital malformations.
Stocking reagents, cleaning, sterilization of equipment, and radiation safety monitoring.

National and International Scientific Participation

1. Journal Editorial Boards

   2018 – 2022, Editorial Committee, Birth Defects Research

   Senior Editor: Michel Vekemans

   Online ISSN:2472-1727; Wiley Periodicals, Inc.

2. Professional Societies

   Developmental Neurotoxicology Society (Member, 2022)

   Society for Birth Defects Research and Prevention (The Teratology Society, Member, 2016)

   Folate Receptors and Transporters Society (Member, 2012)

Patents

1. **Methods of Detecting Anti-Folic Acid Antibodies and Uses Thereof**
   (Pending, Publication number WO2017218632)
2. **Methods of Making Low Odor Choline Salts of an Organic Compound**
   (Publication number WO2018002888A1)
3. **Synthetic Serous Membranes and Methods for Making the Same**
   (Publication number US20120283843 A1)
4. **Methods and Compositions for Oxidizing Bisphenol A**
   (Publication number US20130157336 A1)
5. **Enzymatic Coatings and Methods for Degrading Bisphenol A**
   (Publication number WO2013095350 A1)
6. **Methods and Compositions for Reducing Bisphenol A Release**
   (Publication number WO2014011166 A1)

Publications

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2. Cao X, Xu J, Lin YL, Cabrera RM, Chen Q, Zhang C, Steele JW, Han X, Gross SS, Wlodarczyk BJ, Lupski JR, Li W, Wang H, Finnell RH, Lei Y. Excess folic acid intake increases DNA de novo point mutations. Cell Discov. 2023 Feb 28;9(1):22. doi: 10.1038/s41421-022-00512-0. PMID: 36849450; PMCID: PMC9970956.
3. Han X, Cao X, Cabrera RM, Pimienta Ramirez PA, Zhang C, Ramaekers VT, Finnell RH, Lei Y. KDM6B Variants May Contribute to the Pathophysiology of Human Cerebral Folate Deficiency. Biology (Basel). 2022 Dec 31;12(1):74. doi: 10.3390/biology12010074. PMID: 36671766; PMCID: PMC9855468.

4. Han X, Cao X, Aguiar-Pulido V, Yang W, Karki M, Ramirez PAP, Cabrera RM, Lin YL, Wlodarczyk BJ, Shaw GM, Ross ME, Zhang C, Finnell RH, Lei Y. CIC missense variants contribute to susceptibility for spina bifida. Hum Mutat. 2022 Dec;43(12):2021-2032. doi: 10.1002/humu.24460. Epub 2022 Sep 12. PMID: 36054333; PMCID: PMC9772115.
5. Steele JW, Lin YL, Chen N, Wlodarczyk BJ, Chen Q, Attarwala N, Venkatesalu M, Cabrera RM, Gross SS, Finnell RH. Embryonic Hypotaurine Levels Contribute to Strain-Dependent Susceptibility in Mouse Models of Valproate-Induced Neural Tube Defects. Front Cell Dev Biol. 2022 Feb 21;10:832492. doi: 10.3389/fcell.2022.832492.
6. Tian T, Lai X, Xiang K, Han X, Yin S, Cabrera RM, Steele JW, Lei Y, Cao X, Finnell RH, Wang L, Ren A. Hypermethylation of PI3K-AKT signalling pathway genes is associated with human neural tube defects. Epigenetics. 2022 Jan-Feb;17(2):133-146. doi: 10.1080/15592294.2021.1878725. Epub 2021 Feb 17.
7. Cao X, Wolf A, Kim SE, Cabrera RM, Wlodarczyk BJ, Zhu H, Parker M, Lin Y, Steele JW, Han X, Ramaekers VT, Steinfeld R, Finnell RH, Lei Y. CIC de novo loss of function variants contribute to cerebral folate deficiency by downregulating FOLR1 expression. J Med Genet. 2021 Jul;58(7):484-494. doi: 10.1136/jmedgenet-2020-106987. Epub 2020 Aug 20.
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10. Taiwo TE, Cao X, Cabrera RM, Lei Y, Finnell RH. Approaches to studying the genomic architecture of complex birth defects. Prenat Diagn. 2020 Aug;40(9):1047-1055. doi: 10.1002/pd.5760. Epub 2020 Jun 29.
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12. Cao X, Tian T, Steele JW, Cabrera RM, Aguiar-Pulido V, Wadhwa S, Bhavani N, Bi P, Gargurevich NH, Hoffman EN, Cai CQ, Marini NJ, Yang W, Shaw GM, Ross ME, Finnell RH, Lei Y. Loss of RAD9B impairs early neural development and contributes to the risk for human spina bifida. Hum Mutat. 2020 Apr;41(4):786-799. doi: 10.1002/humu.23969. Epub 2020 Jan 16. Erratum in: Hum Mutat. 2021 Apr;42(4):487.
13. Steele JW, Bayliss S, Bayliss J, Lin YL, Wlodarczyk BJ, Cabrera RM, Asfaw YG, Cummings TJ, Finnell RH, George TM. Heritable spina bifida in sheep: A potential model for fetal repair of myelomeningocele. J Pediatr Surg. 2020 Mar;55(3):475-481. doi: 10.1016/j.jpedsurg.2019.06.019. Epub 2019 Jun 29.
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15. Cabrera RM, Souder JP, Steele JW, Yeo L, Tukeman G, Gorelick DA, Finnell RH. The antagonism of folate receptor by dolutegravir: developmental toxicity reduction by supplemental folic acid. AIDS. 2019 Nov 1;33(13):1967-1976.
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Presentations

2022 - University Hospital Zürich – Clinical Pharmacology and Toxicology Interdepartmental Seminar – Zürich, Switzerland - *Pharmaceutical, Metabolic, and Immunologic Interactions of Folate Receptor*

2022 – Folate in Health and Disease Mini-Symposium – Zürich, Switzerland - *Folate Receptor Autoantibodies: To Thine Own Antigen be True*

2022 - Human Genetics Center Seminar – UT Health Science Center, School of Public Health - Houston, TX - *Gene Environment Interactions in the Etiology and Prevention of Birth Defects*

2021- American Epilepsy Society – Chicago, IL - *Modeling and Prevention of Valproic Acid Induced Birth Defects*

2020 – FASEB Folic Acid, Vitamin B12 & One Carbon Metabolism – Virtual - *Antivirals and Folates: Risky Interactions*

2020 – Teratology Society 60th Annual Meeting – Virtual - *Antivirals and Folates: Risky Interactions*

2019 – Teratology Society 59th Annual Meeting – San Diego, CA - *A Plausible Causative Mechanism for the Developmental Toxicity of Dolutegravir*

2019 – 11th International Neural Tube Defects Conference – Boston, MA - *A Plausible Mechanism for Neural Tube Defects due to Dolutegravir Exposure*

2019 – National Institute for Child Health and Human Development (NICHD)- Neural Tube Defects (NTD) with Integrase Strand Inhibitor Antiretroviral (INSTI) Exposure During Pregnancy Meeting - Bethesda, MD - *Integrase Inhibitor Antagonism of Folate Receptor and Developmental Toxicity Reduction by Folate Supplementation*

2018 – The 5th International Symposium on Folate Receptors and Transporters – Taormina, Italy - *Risky Interactions: Dolutegravir & Folate*

2018 – FASEB Folic Acid, Vitamin B12, and One-Carbon Metabolism - Nova Scotia, Canada - *Elucidation of Folate Mediated Cascades in the Developing Neural Tube: Congenital Malformations Secondary to Methyltransferase Inhibition*

2017 – Children's Environmental Health Institute Ninth Biennial Scientific Symposium - Emerging Science of Endocrine Disruptors and the Unborn Child – Austin, TX - *How Relevant is Food as a Pathway to EDC Exposure?*

2017 – Teratology Society 57th Annual Meeting – Denver, CO - *Fine Mapping for Genetic Susceptibility to Valproic Acid-Induced Exencephaly in Mice*

2016 – 6th International Folate Receptors Symposium - Breckenridge, CO - *Folates That Lie down with Folate Receptor Autoantibodies, Shall Rise up with Antibodies*

2016 – FASEB Folic Acid, Vitamin B12, and One-Carbon Metabolism - Steamboat, CO - Health *Risks of Antibodies to Folic Acid -Poster*

2016 – Teratology Society 56th Annual Meeting – Austin, TX - *Sertraline (Zoloft®) Induced Craniofacial Malformations: Impact of SSRI Exposure on Neural Crest Stem Cells* -Poster

2013 – 8th International Neural Tube Defects Conference – Austin, TX - *Use of Mouse Embryonic Stem Cells as an in vitro Model for Developmental Toxicity Evaluation of Environmental and Commercial Chemicals* -Poster

2012 – Folic Acid, Vitamin B12 & One Carbon Metabolism - Crete , Greece - *Proton-Coupled Folate Transporter and Tissue Uptake of Folates*

2012 – Tools & Technologies in Stem Cells & Regenerative Medicine – Edinburgh, Scotland – *Assessing Developmental Toxicity using Mouse Embryonic Stem Cells*

2012 – ChemScreen Symposium – Amsterdam, Netherlands - *Mouse Embryonic Stem Cell models for Environmental Pollutant Testing*

2011 – 7th International Neural Tube Defects Conference – Austin, TX - *Transcriptional analyses of two mouse models of spina bifida* – Poster

2009 – EPA STAR - Houston, TX - *Mouse embryonic stem cells as a model to elucidate the morphological and mechanistic effects of environmental pollutants.*

2009 – 6th International Neural Tube Defects Conference - Burlington, Vermont. – *Autoantibodies to folate receptor α during early pregnancy and risk of neural tube defects in Norway*.

2008 – Folate Receptors and Transporters – Villa Vigoni, Como, Italy - *Proton-Coupled Folate Transporter Knockout Mice: Hematologic Consequences*

2007 – Reproductive Biology Forum – College Station, TX - *Mechanisms of Folic Acid in the Reduction of Neural Tube Defect.*

2005 – 4th International Workshop on Neural Tube Defects - Palm Springs, CA – Award.

2005 – 5th Int. Conference on Homocysteine Metabolism - Milano, Italy - *Elucidation of Folate Mediated Cascades in the Developing Neural Tube: Congenital Malformations Secondary to Methyltransferase Inhibition.*

2005 – 13th Int. Symposium on Chemistry & Biology of Pteridines & Folates - Egmond aan Zee, The Netherlands - *Influence of Folic Acid and Metabolites of Folate Biochemistry on NTD rates* - Award.

2004 – FASEB Summer Research Conference - Folic Acid, Vitamin B12 and One-Carbon Metabolism - Snowmass, CO - *Gene Expression Analysis in the Developing Neural Tube: Elucidation of Folate Mediated Cascades.*